PB85-165801

Microbial Toxicity Studies

(U.S.) Environmental Research Lab.
Gulf Breeze, FL

Feb 85

U.S. Department of Commerce
National Technical Information Service
NTIS

PB85-165801

EPA/600/D-83/027
February 1985

MICROBIAL TOXICITY STUDIES

by

P. H. Pritchard and A. W. Bourquin

Environmental Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Gulf Breeze, Florida 32561

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA. 22161

TECHNICAL REPORT DATA (Please read Instructions on the reverse before completing)		
1. REPORT NO. EPA/600/D-83/027	2.	3. RECIPIENT'S ACCESSION NO. PB85 165801 /AS
4. TITLE AND SUBTITLE AQUATIC MICORIBAL TOXICITY STUDIES		5. REPORT DATE February 1985
		6. PERFORMING ORGANIZATION CODE
7. AUTHOR(S) P.H. Pritchard and A.W. Bourquin		8. PERFORMING ORGANIZATION REPORT NO.
9. PERFORMING ORGANIZATION NAME AND ADDRESS same as #12		10. PROGRAM ELEMENT NO.
		11. CONTRACT/GRANT NO.
12. SPONSORING AGENCY NAME AND ADDRESS U.S. Environmental Protection Agency Environmental Research Laboratory Office of Research and Development Gulf Breeze, FL 32561		13. TYPE OF REPORT AND PERIOD COVERED
		14. SPONSORING AGENCY CODE EPA/600/04
15. SUPPLEMENTARY NOTES		

16. ABSTRACT
This chapter cites examples of the common methods used to determine the toxicity of chemicals to bacteria. It covers only the most common methods, particularly those that are easy to perform. Numerous literature citations have been included to help illustrate how a method is used and where its advantages and disadvantages lie. The information presented here is not a complete survey, but rather an aid to understanding of current methods used in the field of microbial toxicology. Toxicity of chemicals to bacteria is discussed relative to the organisms' growth and metabolism. Examples are given of methods involving heterotrophic activity, geochemical cycling, and decomposition processes. In addition, application of toxicity studies to unique bacterial processes and habitats discussed.

17. KEY WORDS AND DOCUMENT ANALYSIS		
a. DESCRIPTORS	b. IDENTIFIERS OPEN ENDED TERMS	c. COSATI Field Group

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS (This Report)	21. NO. OF PAGES
Release to public	unclassified	46
	20. SECURITY CLASS (This page) unclassified	22. PRICE

EPA Form 2220-1 (Rev. 4-77) PREVIOUS EDITION IS OBSOLETE

NOTICE

This document has been reviewed in accordance with U.S. Environmental Protection Agency policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

TABLE OF CONTENTS

I. Introduction

II. Terminology

III. General Background in Aquatic Microbiology

IV. Effect of Toxicants on Microbial Growth

- A. Enumeration of Bacteria in Aquatic Environments
- B. Growth of Heterotrophic Microorganisms
- C. Toward a Standard Bacterial Toxicity Test

V. Effects of Toxicants on Microbial Activities

- A. Heterotrophic Activity
- B. Geochemical Cycling - Nitrogen
- C. Decomposition Processes

VI. Unique Aspects of the Effects of Toxicants on Microorganisms

- A. Chemotaxis
- B. Epiphytic Microorganisms
- C. Incidence of Plasmids Containing Bacteria
- D. Bioaccumulation in Bacteria

VII. Adverse Effects of Microorganisms on Toxic Substances

VIII. Summary

IX. Suggested Reading

X. Literature Cited

INTRODUCTION

Toxicologists and microbiologists have been concerned with the effects of toxic materials on microbial growth and activities for many years. Studies of terrestrial microorganisms have been particularly plentiful, since the success of microbial geochemical events is very much a part of successful crop production. However, there have also been numerous studies of aquatic microorganisms because of the increasing incidence of toxicants in aquatic systems. Although the disruption of microbial activities could be catastrophic for an ecosystem, it has been found that in general bacteria are the most insensitive organisms to toxicants. In fact, there are very few reported cases of chemical toxicity to microorganisms at a concentration below that which adversely affects higher animals. One of the few cases is the compound N-Serve (Billen, 1975) developed specifically to inhibit denitrification in soil. This chemical is unique because it affects a very special bacterial process.

The relative insensitivity of bacteria to toxicants is due primarily to bacterial metabolic processes. Bacteria are small organisms whose metabolic processes have been reduced to a minimum so that they efficiently utilize carbon and energy sources and can reproduce rapidly. The simplicity of their metabolism and macromolecular organization is generally not characteristic of higher organisms, in which metabolic and structural complexity and specialization are commonplace. It is often because of this specialization that certain higher organisms are more sensitive to toxicants. In fact, many pesticides are designed to affect specific target organisms (Chapter 12) by disrupting one of these specialized processes or

structures. A good example is the pesticide Dimilin, which specifically inhibits chitin synthesis. Although Dimilin is effective against insects, it also affects other organisms such as crustaceans, which synthesize chitin. As another example, organophosphate insecticides are designed to inhibit the enzyme acetylcholinesterase and nerve transmission, but bacteria, which have no enzyme system similar to that of insects, are relatively insensitive to organophosphates. Conversely, chemicals that are general inhibitors of metabolic processes common to all types of organisms are capable of adversely affecting bacteria.

Another important factor in the relative insensitivity of bacteria to toxic chemicals is the rather loose connection between disruption of cellular processes and cell death. Bacteria can become dormant and survive adverse conditions, whereas higher organisms generally lack this ability. In general, once the liver of a fish is adversely affected, the animal dies quite rapidly. Since bacteria have relatively few specialized organs or metabolic processes, death of the organism after impairment of a function may not occur for a considerable time. Likewise, if growth conditions are not ideal for a particular bacterium, it can slow down its growth and direct its energy to simply surviving. Frequently, the effect of chemical exposure on bacteria is biostatic (inhibiting cell growth but not killing cells) rather than biocidal (killing cells), and thus recovery of a normal population of bacteria following chemical exposure is quite high.

Finally, it appears that bacteria provide minimal access for a toxicant. Their transport mechanisms are very selective, and many toxicants are never transported into the cell. Bacteria lack gills, digestive tracts, livers, kidneys, mitochondria, and other typical organs and systems and consequently are far less likely to absorb a toxicant into the cell. Also, the complex bacterial cell wall and associated enveloping slime provide a more protective coating than, for example, the gut wall of an invertebrate.

Even if a toxicant is shown to have an adverse effect on a bacterium or a bacterial population, the environmental significance of such an effect is very difficult to assess for a number of reasons. First, bacteria grow relatively rapidly and can thus quickly regain their original density if only part of the population is killed. Second, in most cases bacterial specialization is in metabolic capabilities rather than structural aspects, and many bacteria of different genera carry out the same metabolic processes. If a prominent species in a population is eliminated, chances are good that a previously minor species will elaborate and adequately fill the temporary metabolic void. Therefore, since the same general metabolic processes are widespread among so many bacteria, it is unlikely that a toxicant will have a long-term effect. There are some unique metabolic processes that must be considered, however. For example, the types of bacteria associated with nitrification are very limited and are very slow growers. If these bacteria are specifically affected by a toxicant, functional recovery could be quite slow and the geochemical cycling of nitrogen could be impeded. Therefore, nitrifying bacteria constitute one group of microorganisms that should be considered first in toxicity assessments. Third, the ubiquity of bacteria and redundancy of metabolic processes within heterogeneous populations mean that localized effects of a toxicant can be compensated in a relatively short time. In aquatic environments, especially, there is great movement and mixing of bacterial populations, and it is quite easy for this exchange to compensate any damage to the population even from a chronic toxicity effect. If large numbers of bacteria were uniquely adapted for survival in a particular aquatic environment, this buffering effect would not be as great—for example, bacteria washed in from terrestrial environments could not replace the aquatic population. In general, this situation does not exist; terrestrial bacteria readily survive and reproduce in aquatic systems.

Despite the relative insensitivity and post-exposure recovery potential of bacterial popula-

tions, these organisms are useful for evaluating the toxicity of new chemicals in the aquatic environment, since this type of screening can lead to early identification of any new and potentially powerful bactericide before extensive environmental contamination has occurred. Certainly, if a chemical is found to be significantly toxic to bacteria, there is a substantial possibility that it may be even more toxic to higher plants and animals.

This chapter gives some examples of common methods used to determine the toxicity of chemicals to bacteria. Only the most common methods, particularly those that are easy to perform, are discussed. Numerous literature citations are included to help illustrate the use of each method and its advantages and disadvantages. The information presented is by no means a complete survey of the field. The supplemental reading list provides considerably more information on these methods.

TERMINOLOGY

Allochthonous. From without. Refers to organisms or organic material that is not produced or sustained within a particular environment.

Ammonification. A subset of mineralization in which organically bound nitrogen is released as ammonia.

Autochthonous. From within. Indigenous organisms or organic material.

Axenic culture. A pure culture, one free of other organisms.

Autotrophic. Obtaining carbon from the fixation of carbon dioxide and energy from the oxidation of specific inorganic compounds.

Bactericidal. Producing mortality; generally used in relation to toxic chemicals and disinfectants.

Bacteriostatic. Preventing growth and proliferation of bacterial cells without killing them.

Denitrification. The process of converting nitrogen compounds to molecular nitrogen (N_2).

Epifluorescence. The observation of fluorescent light from hydrogen and halogen lamps.

Facultative anaerobes. Bacteria that can grow in the presence or absence of oxygen.

Gnotobiotic. Composition completely known, often referring to mixed populations of organisms.

Gram-negative or gram-positive. A general classification of bacteria used on the differential staining procedure developed by Hans Gram.

Heterotrophic. Requiring preformed organic substances for carbon and energy.

K_S. A constant referring to the concentration of substrate that will produce half the V_{max} (see below). Generally reflects the affinity of a catalytic unit (enzyme, cell, etc.) for a substrate.

Lipopolysaccharide. A chemical substance made of specific lipids and sugars that form the external surface of gram negative bacteria.

Methanogenic. Producing methane gas.

Mineralization. The biological process of transforming organic matter by complete oxidation into carbon dioxide, water, and other inorganic compounds.

Muramic acid. The chemical backbone of bacterial cell walls; unique to bacteria.

Nitrification. The process of converting ammonia to nitrite and nitrate.

Nitrogen fixation. The process of fixing molecular nitrogen into organic matter.

Obligate anaerobes. Bacteria that cannot grow in the presence of oxygen.

Plasmids. Extrachromosomal nonnuclear, self-replicating units of DNA that are not an essential part of the cell but can carry genetic information.

V_{max}. Maximum velocity (rate) of an enzyme-catalyzed reaction or biological process.

GENERAL BACKGROUND OF AQUATIC MICROBIOLOGY

Bacteria in aquatic environments can be found in very diverse habitats and communities. They are associated with all types of surfaces, including plants, rocks, animals, sediment, manufactured objects, and plankton, and they are found in

chemical ranges: temperature −4 to +50°C, salinity 0 to 100 ppt, pressure 0 to 1000 atm, pH 5.5 to 8.5, and oxidation/reduction potential (Eh) +400 to −400 mV. The numbers, types, and activities of these bacteria are basically dictated by their environmental setting. Concentrations of organic and inorganic materials and temperature are probably the most restrictive factors controlling the growth of bacteria in aquatic habitats. Most bacteria are heterotrophs, deriving carbon and energy for growth from the dead remains of plant and animal material. Dissolved organic food material from these remains is often found in extremely low concentrations (μg/l) in aquatic habitats; thus, aquatic bacteria are efficient scavengers (Rheinheimer, 1971). Bacterial metabolism is geared to the utilization of small concentrations of nutrients that appear intermittently.

In the water column, bacteria are found free-swimming and attached. Those that attach often do so to themselves (aggregates), to inert organic and inorganic particulate matter, or to the surfaces of phytoplankton and zooplankton. In general, suspended particles have a favorable effect on microbial growth because they are focal points for adsorption and desorption processes that provide nutrients for attached bacteria and they neutralize toxic effects of other compounds.

Bacteria associated with sediment and with plant and animal surfaces are often found in greater numbers per unit area than those that are free-swimming. However, overall microbial activity may not reflect this mass difference because sediment and surface bacteria cells may be packed several cell layers thick. Those on the bottom layers are shielded from available nutrients and live essentially as dormant populations.

Aquatic environments typically have a distribution of bacterial populations in which cell numbers decrease with distance from shore and with depth. There are exceptions, of course. Surface slicks on lakes and bays are frequently rich in bacterial biomass and metabolic activities compared to the water immediately below. The same is true of the sediment-water interface, where a loose slurry of sediment (turbidity layer) may contain many of the mineralization activities associated with the aquatic environment. Thermoclines and euphotic zones of lakes and oceans can also have areas with greater bacterial numbers and sometimes greater bacterial activities.

The principal activity of bacteria is the transformation of organically bound carbon, nitrogen, phosphorus, magnesium, sulfur, and other elements into unbound oxidized states (nitrate, sulfate, phosphate). This process is called mineralization, and it is the key to nutrient cycling in aquatic systems. These nutrients provide plants with the essential factors needed for growth. Thus, toxicologists and microbiologists are concerned with the effects of toxic materials on bacteria and the processes they mediate. If mineralization and nutrient recycling become severely disturbed or curtailed, the functioning of the ecosystem will be dramatically affected since the two are so closely linked. Domestic sewage, for example, severely affects the microflora of waters. It changes the normal pattern of mineralization and has undesirable aesthetic and economic consequences. The massive increase in nutrients associated with sewage pollution causes algal blooms. The algae eventually die, leaving a large pool of readily degradable organic material which the bacteria attempt to eliminate through mineralization. Invariably, in heavily affected areas bacterial degradative activities become limited, and with oxygen depletion, large concentrations of sulfide accumulate, killing fish and other animals. Specific toxicants can have similar effects; thus it is important to routinely assess potential impacts on aquatic bacteria and the processes they catalyze.

Effects of Toxicants on Microbial Growth

Population growth is relatively easy to measure in the laboratory. It can be studied in natural mixed populations, synthetic gnotobiotic populations, or pure cultures. Some researchers consider that pure cultures are preferable for toxicity studies in certain situations; others think that mixed populations should be studied.

Growth studies with bacteria are generally simple and can employ a variety of experimental conditions related to specific physical and chemical characteristics of aquatic environments. Microbial populations spanning a range of physiological conditions and ecosystem types are easily accessible. Samples of sediments, soil, water, plants, and other material can be transported to the laboratory. With little additional effort, growth of microbial populations can be observed under laboratory conditions and the effect of a toxicant measured. Thus, initial estimates of toxicity invariably involve growth studies.

Enumeration of Bacteria in Aquatic Environments

To perform toxicity studies on bacteria it is necessary to be able to enumerate the bacteria in a specific population from a specific habitat and quantitatively measure metabolic activity. Many applicable techniques are discussed in subsequent sections. Because of their size, bacteria present unique problems that may make toxicity studies difficult to interpret.

For example, live bacterial cells are commonly enumerated by spreading a dilute water sample on an agar medium that will support growth. If individual cells are separated sufficiently from their neighbors, they will grow and divide, eventually producing a visible colony. The number of colonies equals the number of bacteria present in the diluted water sample. This is a mechanically simple exercise, but the interpretation can be confusing. First, there is no guarantee that a colony originated from a single cell. It may have aggregated during or prior to plating. If the toxicant provoked aggregation, the result would seem to be a reduction in cell numbers and an apparent toxicant-mediated mortality. Second, a toxicant may have a bacteriostatic effect, so that once it is washed away, the effect disappears. Enumeration techniques involving agar often ensure a constant concentration of toxicant, resulting in a misleading reduction in numbers not seen in nature. Third, the agar medium should allow growth of all the bacteria in the water sample. In most situations, however, only a small percentage of the total bacteria in the sample will grow on a particular medium. With the diversity of metabolic types in natural bacterial populations, it is virtually impossible for all of the types to grow on a single type of medium. Thus, in a toxicity test, one never knows whether the bacteria that did not grow on a particular medium were more sensitive to the toxicant.

An alternative to counting viable cells on agar is counting bacteria directly with a microscope. This technique has been improved by the advent of epifluorescence counting (Daley and Hobbie, 1975). Cells are stained with acridine orange, a fluorescing dye that associates tightly with DNA and can be observed under a fluorescence microscope. The bacteria stand out vividly from the background, and all bacteria in the sample can easily be counted. However, many bacterial cells can remain intact even though functionally dead. Consequently, it is difficult to distinguish the live from the dead cells, which makes interpretation of a toxicity study difficult. Several variations on the epifluorescence technique have been used to differentiate live from dead bacteria. Nalidixic acid, for example, interferes with RNA synthesis in growing cells, causing them to enlarge and change shape, and this allows live bacteria to be distinguished morphologically from dead cells (Orndorff and Colwell, 1980). This technique may prove useful in toxicity tests with bacteria.

Direct counts invariably yield higher numbers of cells per unit volume than do plate counts, because dead cells and metabolically dormant cells are all counted together. However, direct counting has advantages, particularly in relation to microbial activity measurements, as will be shown in more detail below. Direct counts of bacteria are reasonable only if the numbers are generally higher than 10^3 or 10^4 cells per milliliter. At lower concentrations, the chance of detecting cells in a microscopic field is low. When cell concentrations are low, other counting techniques (such as plate counts) must be employed.

Several other methods for enumerating bacteria are also available, but their application to toxicity testing is limited. Bacterial growth in broth media causes increased turbidity, which can be quantitated with a spectrophotometer. The turbidometric method has been used extensively in microbiology, particularly with pure cultures. It is a very simple, reproducible technique. The classical multiphase growth response of bacterial cultures [lag phase (no growth), logarithmic growth phase (maximal growth), stationary phase (nutrient depletion), and death phase (cell lysis)] can be routinely assessed. This technique is also valuable when studying the phases of growth that are most sensitive to a toxicant. However, growth phases are essentially a laboratory artifact; caution is required in extrapolating the results of the field.

Several biochemical components of bacterial cells can be quantitated and related to cell numbers. Measurements of cellular ATP levels, cell wall components such as lipopolysaccharide or muramic acid, lipid phosphate and other lipid components, nuclear material such as DNA and RNA, and certain enzyme levels have been used with moderate success. In most cases, except for muramic acid and lipopolysaccharide, the measurements are not unique to bacteria; the presence of one algal cell or protozoan can greatly affect the concentration of these components and produce very misleading estimates of bacterial population densities. Muramic acid, which is found in the cell walls of cyanobacteria (blue-green algae), often gives misleading information, since many cyanobacterial cells are considerably larger than most bacteria.

In toxicity testing with microorganisms, it is important to be aware of the limitations of studies that concentrate on effects measurements related to changes in bacterial cell concentration or to mortality based on morphology rather than physiology. In other types of toxicity studies, a dead *Daphnia* or fish is obvious; the same clear-cut observation is not always possible with bacteria. In this chapter this limitation will be illustrated, and it will be shown how physiological indices, which represent a new and subtle approach to toxicity testing in higher organisms, are excellent effects-related parameters for bacterial populations.

Growth of Heterotrophic Microorganisms

The most common way to study the effects of toxic materials on microorganisms is to monitor growth inhibition on agar media by a viable plate count. Dilutions of natural water samples are plated on agar media that contain different concentrations of the toxicant, and the reductions in colony forming units (CFUs) relative to control plates containing no toxicant are then observed. The plate count technique is based on the principle that each viable organism will give rise to one colony. This method is simple, economical, suitable for statistical analysis, and amenable to the examination of large numbers of water samples. Numerous reports on this type of study are available (see Ware and Roan, 1970).

An example of this type of study is work on the toxicity of Kepone to estuarine microorganisms (Mahaffey et al., 1982; Bourquin et al., 1978). Kepone is an organochlorine pesticide that contaminated the James River estuary in Virginia. To determine its toxicity to microorganisms, samples of estuarine water from several sampling sites around Pensacola, Florida, were serially diluted and plated on Zobell's agar containing Kepone concentrations of 0.1-2.0 mg/l (ppm). A significant reduction ($\alpha = 0.05$) in CFUs relative to control plates was detected on plates containing 0.2 mg/l (Table 1). Twenty-three different colonies were selected from these plates and developed as pure microbial cultures. The physiological characteristics of these isolates are detailed in Table 2. These isolates, overall, were found to be sensitive to Kepone to varying degrees, but gram-negative isolates were generally least sensitive to this pesticide.

Since Kepone in the James River is primarily found in the sediments, it was important to determine whether the anaerobic organisms found primarily in estuarine sediments were also affected by Kepone. Kepone was not nearly as toxic to anaerobically grown microorganisms (Table 3).

Table 1 Effects of Kepone on Isolation of Aerobic Bacteria from Estuarine Water [a]

Sample source	Date[b]	Salinity (‰)	Temperature (°C)	CFUs x 10^3 per milliliter of water			
					Kepone[c] concentration (mg/l)		
				Control	0.02	0.20	2.00
Range Point	A	15	21	181.0	108.0 (41)[d]	73.0 (60)	35.0 (81)
	B	15	24	86.5	65.0 (25)	35.0 (60)	33.0 (61)
	C	20	23	17.7	12.0 (28)	13.0 (23)	13.0 (25)
Escambia Bay	A	11	22	267.0	81.0 (70)	233.0 (13)	130.0 (52)
	B	15	24	13.5	6.9 (50)	10.0 (26)	4.7 (66)
	C	18	22	11.6	11.0 (8)	9.0 (23)	8.9 (23)
Laboratory pond	A	20	20	479.0[e]	448.9 (7)[e]	409.0 (14)[e]	396.0 (18)[e]
	B	15	26	104.0	49.0 (53)	–	19.0 (82)
	C	20	27	544.0	501.0 (8)	437.0 (20)	309.0 (43)
Gulf of Mexico	A	33	18	270.0	62.0 (78)	32.0 (89)	46.0 (83)
	B	33	22	13.3	10.5 (21)	10.0 (25)	8.0 (40)
	D	33	24	15.0	6.1 (59)	1.2 (92)	0.7 (95)

[a] From Mahaffey et al. (1982).
[b] Sampling date (1977): A, April 1; B, April 17; C, April 29; D, May 7.
[c] Kepone added to Zobell's marine agar.
[d] Numbers in parentheses are percent reduction in CFUs normalized against growth on control plates of Zobell's marine agar containing no Kepone; all percent reductions are significant ($\alpha = 0.05$).
[e] CFUs x 10^3 per milliliter.

This difference is probably due to interference of Kepone with oxidation and respiration, similar to the mechanism described by Widdis et al. (1971) and Trudgill et al. (1971) for the toxicity of chlordane to *Bacillus* sp.

Orndorff and Colwell (1980) compared the viable plate counting technique with direct counting methods. The acridine orange epifluorescence method developed by Daley and Hobbie (1975) was used for direct counts of bacterial populations. In this method, acridine orange stains biological material and gives a distinct green color under the fluorescence microscope. To obtain an indication of the number of live bacteria comprising the total direct count, Orndorff treated the cells with nalidixic acid by the method of Kogure (1979). Nalidixic acid specifically inhibits RNA synthesis in live gram negative cells and causes a change in their shape, enabling them to be distinguished from dead cells. Since most aquatic microorganisms are gram-negative, and Kepone is not toxic to gram-negatives, use of the Kogure technique is justified.

Results of Orndorff and Colwell (1980) in Table 4 indicate that viable direct counts (by fluorescence microscopy) comprised 2.5-56.7% of the total direct counts (mean $\bar{x} = 24.5\%$) and counts on the agar plates were 0.5-2 orders of magnitude less than the viable direct counts. It appears that the concentration-response curve is about the same, regardless of the counting method. The viable direct count is probably the most accurate method, yet the same relative toxicity can be established with the simpler viable plate counts.

In all these studies it is important to perform a statistical analysis on the data to determine whether effects resulting from a toxicant exposure are significant. Several statistical analyses can be used for this purpose. For example, in the work by

Table 2 Physiological Activities of Kepone-sensitive and Kepone-tolerant Microbes[a]

Culture number	Organism type	Gram stain	Alkane utilizer[b]	Aromatic utilizer[b]	Physiological function			Pesticide tolerance[c]
					Lipolytic	Amylolytic	Proteolytic	
				Kepone-sensitive				
1	Rod	Positive	+	+	–	+	+	+
3	Rod	Positive	–	–	–	+	+	–
4	Cocc-bac	Negative	–	–	+	+	+	–
11	Cocc-bac	Positive	+	+	+	+	–	+
14	Rod	Positive	–	–	+	+	–	–
29	Rod	Positive	–	–	–	–	+	–
42	Coccus	Positive	–	–	+	–	–	–
54	Rod	Positive	–	+	–	+	+	–
8	Yeast	–	–	+	+	+	+	–
15	Fungus	–	+	+	ND[d]	ND	ND	–
				Kepone-tolerant				
10	Rod	Negative	–	–	–	–	±	–
18	Pleo	Negative	+	–	+	–	–	–
20	Rod	Negative	+	–	ND	ND	ND	–
23	Rod	Positive	+	+	ND	ND	ND	+
40	Cocc-bac	Positive	+	–	+	–	+	+
45	Rod	Negative	–	–	+	–	+	–
46	Rod	Negative	–	–	+	–	–	+
56	Cocc-bac	Negative	–	–	+	+	–	+
59	Rod	Negative	–	–	–	–	–	–
2	Yeast	–	+	–	–	–	+	–
7	Yeast	–	–	–	+	+	+	–
27	Fungus	–	–	+	ND	ND	ND	+
44	Fungus	–	–	–	ND	ND	ND	+

[a] From Bourquin et al. (1978).
[b] Hydrocarbons tested were hexadecane, undecane, octadecane, benzene, naphthalene, biphenyl, xylene, and toluene.
[c] Pesticide tolerance test means growth in the presence of toxaphene, Aroclor 1242, methoxychlor, heptachlor, DDT, malathion, and pentachlorophenol.
[d] ND, not determined.

Table 3 Colony-forming Units in Sediments[a,b]

Date (1977)	Type	Kepone concentration (mg/l)			
		0	0.02	0.2	2.0
August 2	Aerobic	207.50 ± 22.00	197.50 ± 41.00	13.75 ± 13.00	33.20 ± 16.00
	Anaerobic	34.00 ± 4.30	30.60 ± 10.00	41.00 ± 10.00	36.25 ± 3.10
August 9	Aerobic	235.00 ± 75.00	74.00 ± 17.00	53.70 ± 18.00	26.25 ± 10.00
	Anaerobic	76.75 ± 12.70	68.25 ± 20.30	67.50 ± 25.00	59.50 ± 23.00
August 31	Aerobic	215.75 ± 51.30	243.50 ± 98.60	207.50 ± 99.50	62.25 ± 56.00
	Anaerobic	26.25 ± 7.00	24.50 ± 3.40	18.00 ± 8.00	13.25 ± 1.50

[a] From Mahaffey et al. (1982).
[b] Cells X 10^2 per milliliter, average of four replicates.

Table 4 Enumeration of Microbiological Populations from Colgate Creek, Maryland, in Water Samples with Different Concentrations of Kepone[a]

Sampling[b] data	TVC[c]	TC[d]	DVC[e] Kepone concentration (mg/l) 0	0.01	0.05	0.2
October	3.1×10^5	2.0×10^6	5.4×10^5	3.9×10^5	3.1×10^5	4.2×10^4
November	1.9×10^5	2.7×10^6	1.2×10^6	2.2×10^5	2.8×10^5	1.8×10^5
December	9.9×10^4	1.5×10^7	3.9×10^6	3.9×10^6	1.8×10^6	1.3×10^5
March	5.3×10^5	2.3×10^6	2.3×10^6	2.8×10^6	1.3×10^6	4.9×10^5

[a] From Orndorff and Colwell (1980).
[b] One sample was collected each month during 1978–1979.
[c] TVC, total viable heterotrophs per milliliter determined by plate count method.
[d] TC, total count per milliliter determined by acridine orange direct counting.
[e] DVC, direct viable counts per milliliter.

Mahaffey et al. (1982) the effect of Kepone on plate counts of bacteria was statistically analyzed by a three-factor model I analysis of variance (Zar, 1974). A comparison was made of 150 observations and 4 factors: Kepone concentration, aerobic versus anaerobic incubation, sampling date, and an air-treatment interaction component. A Student-Newman-Keuls (SNK) post hoc analysis was performed to evaluate the significant differences between population means. This test was used to constrast the Kepone-dependent reduction in CFUs under aerobic and anerobic conditions. Figure 1 shows the mean number of CFUs for aerobic and anaerobic incubations plotted against

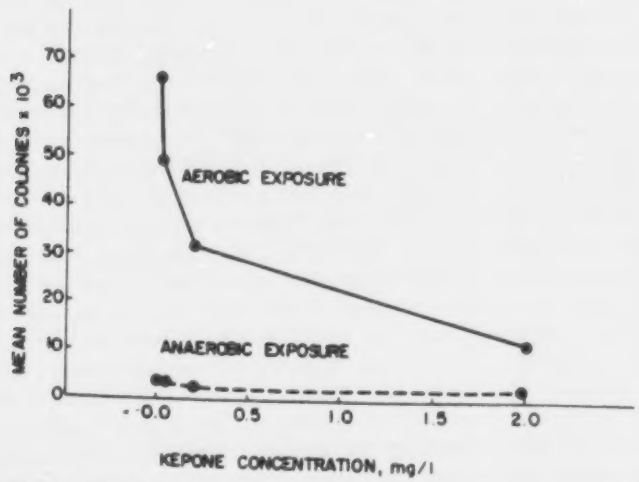

Figure 1 Effect of Kepone on the growth of natural marine microbial population under aerobic and anaerobic conditions. Control levels of colonies are shown at the zero concentration. *[From W. R. Mahaffey, P. H. Pritchard, and A. W. Bourquin (unpublished data).]*

Kepone concentration. The mean responses to aerobic and anaerobic treatments, on different sampling dates were significantly different at a confidence interval of $\alpha = 0.05$. The SNK post hoc analysis showed that populations grown under anaerobic conditions were not significantly affected by Kepone at any concentration tested. However, significant reductions in CFUs were observed under aerobic conditions. Significant differences ($\alpha = 0.005$) were observed between control populations and those grown aerobically in the presence of 0.2 and 2.0 mg/l Kepone. Differences were also observed between the 2.0 mg/l treatment and 0.02 and 0.2 mg/l treatments, indicating a minimum inhibitory concentration (MIC) of 0.2 mg/l Kepone.

Goulder et al. (1980) used bacterial growth studies to examine the relationships between heterotrophic bacterial populations and industrial pollution. This excellent paper can be used as a model for effect studies with bacteria. Many variables were assessed, including heavy-metal types and concentrations, bacterial activities and population densities, and environmental variables such as oxygen, salinity, organic content, and suspended particulate matter (Table 5). Attached and free bacteria were counted (acridine orange epifluorescence method); viable counts were performed (standard plate counts); and statistical correlations between the bacterial populations in the estuary and a number of system variables (Table 5) associated with the pollution stress were determined. A strong positive correlation was found between attached bacteria or viable counts and suspended particulate matter; yet a negative correlation existed between activity measures of bacterial populations and suspended particulate matter. This suggests that an increase in cell number does not necessarily mean an increase in activity, and degradation rates of these organic pollutants associated with suspended particulates may remain the same regardless of the inputs to the estuary. Numbers of bacteria, both attached and free, and bacterial activity were positively correlated with organic content. This can be interpreted as meaning that no widespread toxic effect existed because there was no correlation between these factors and the presence of toxic materials. The evidence also showed that heavy metals, particularly copper, may have inhibited bacterial growth at high concentrations. The inhibition was noted at an industrial outfall where copper concentrations were high.

The work described above demonstrates a useful methodology for determining the toxic effects of complex effluents. This method could readily be used to assess the potential dangers of an effluent to the microbial ecology of an estuarine environment. The number of determinations performed makes the method labor-intensive, but the measurements are simple and the results may be useful in long-term evaluations.

Fry et al. (1973) showed that monitoring of bacterial populations with viable plate counts was useful in developing a complete assessment of the effects of the herbicide paraquat. After the first application of paraquat to a large reservoir, all the macrophytes were killed. An immediate reduction in the number of heterotrophic bacteria in the water column was observed, but bacteria in the sediments were unaffected. During the second treatment (45 days later) no reduction in heterotrophs was observed, but the number of paraquat-resistant bacteria also increased. This tolerance may have been associated with release of soluble organic material from the dead plants, which also stimulated microbial growth and increased population densities. In fact, many bacteria that grew up during the second treatment were producers of extracellular enzymes such as amylase and protease. The increase in exoenzyme producers was probably a response to the organic material released by the decaying plants. Thus, information could be gained from plate counts and the correlation between cell numbers and the general ecology of this reservoir.

An example of the flexibility of the plate count approach for assessing toxicity to bacteria is presented by Guthrie et al. (1974), who described the natural bacterial population balance in a lake

Table 5 Significance Levels of Correlation Coefficients[a] between Bacterial and Environmental Variables in an Industrialized Estu from East Clough in Humber Estuary, England[b]

	Pollution-independent variable[c]				Indicator of organic pollution[c]						Heavy metals[c]							
Bacterial measurements	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Attached bacteria	3	2	0	3	0	2	−1	0	0	−1	2	0	3	0	3	0	0	0
Free bacteria	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	−3	0	0
Viable bacteria	0	0	0	0	0	2	−1	3	0	0	0	−3	0	−2	0	0	−2	−2
V_{max}	0	0	0	1	0	0	0	1	−1	0	0	−2	0	0	0	0	−2	−2
Turnover time	0	0	0	0	0	0	0	0	1	0	0	2	0	0	0	0	2	2
Attached bacteria/suspended solids	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
V_{max}/bacterium	0	0	0	0	0	0	0	0	−1	0	−2	−3	0	0	0	0	−3	0
Cu-tolerant bacteria	1	0	0	0	0	0	0	0	1	−1	3	0	1	0	2	0	2	0
Zn-tolerant bacteria	0	0	0	0	0	0	1	0	2	0	1	3	0	0	0	1	3	0

[a] 0 indicates no significant correlation ($p > 0.05$). Significant correlations are indicated by 1 ($p < 0.05$), 2 ($p < 0.01$), and 3 ($p < 0.001$). N indicate negative correlations.

[b] Reproduced with permission from Goulder et al. (1980); copyright, Pergamon Press, Ltd.

[c] 1, Suspended solids; 2, salinity; 3, oxygen; 4, suspended organic matter; 5, total permanganate O_2 demand; 6, particulate permanganate O_2 d dissolved permanganate O_2 demand; 8, presumptive *E. coli*; 9, *E. coli*/viable bacteria; 10, organic matter as percent of suspended solids; 11, particula dissolved Cu; 13, particulate Pb; 14, dissolved Pb; 15, particulate Zn; 16, dissolved Zn; 17, Cu/suspended solids; 18, Pb/suspended solids; 19, Zn/ solids.

ecosystem. Samples of water from two different lake environments and from laboratory tank culture systems designed to simulate the lake environments were exposed to the herbicide diuron, and effects on bacterial populations were measured in three different ways. First, plate counts were performed and an overall net decrease in bacterial population was observed. An examination of colony morphology showed that the chromogenic (colored) colonies were significantly reduced. The apparent sensitivity of the chromogenic bacteria to this chemical is difficult to explain.

Second, disk sensitivity tests were performed. A pure culture of a bacterium isolated from the original plate counts was spread on an agar plate. Sterile filter paper disks saturated with different concentrations of toxicant (diuron) in aqueous solution were placed on the plate immediately after spreading. A zone of inhibition around the filter disks indicates toxicity (Fig. 2). The zone of inhibition crudely quantitates the degree of toxicity of the chemical to the bacteria. Surprisingly, many of the isolates, particularly the chromogenic types, were not affected by diuron in the disk sensitivity method. This suggests that a secondary, toxicant-related process affected the viability of the chromogenic forms.

Third, plate counts were performed on an agar medium supplemented with milk, Tween 80 (a surfactant), or starch. Many bacterial species secrete extracellular enzymes capable of hydrolyzing a number of these polymers. This activity

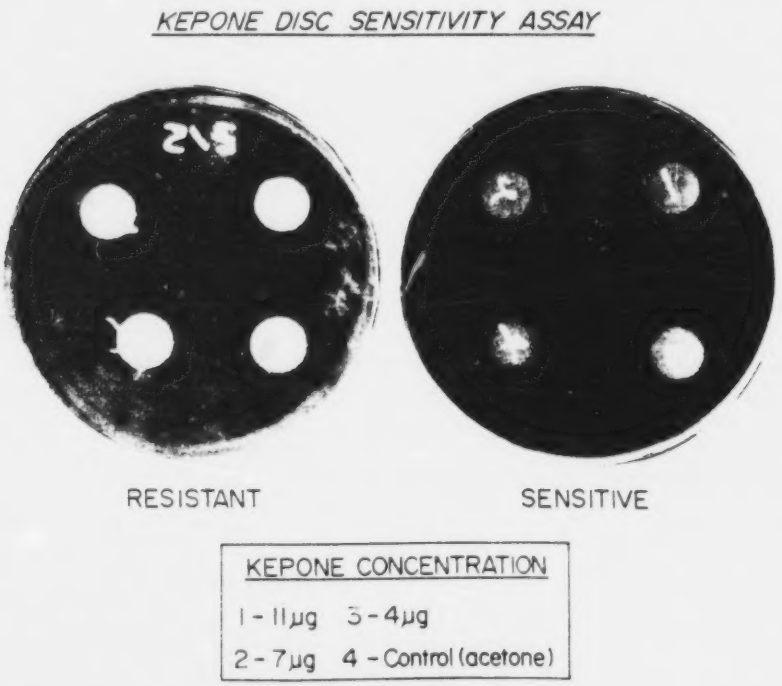

Figure 2 Example of the zone-of-inhibition method for determining toxicity of a chemical to growth of bacteria. Cloudiness on agar surface is microbial growth. White circles are filter paper disks saturated with the toxicant. Zones of clearing around disk indicate area where bacterial growth on agar was inhibited. *[From A. W. Bourquin (unpublished data).]*

results in a change in the consistency of the agar around each colony, and lipid hydrolyzers, starch hydrolyzers, and casein hydrolyzers can be readily detected. A toxicant that is more toxic to one physiological group than to the entire population can be easily observed; thus, a specific different toxic response can be assessed. In Guthrie's study, starch-, lipid-, and protein-hydrolyzing bacteria were present, but they did not show any differential sensitivity to the diuron (Table 6). In a similar study, Mahaffey et al. (1982) selected (on the basis of predominance) a total of 17 CFUs that grew in the presence of Kepone. Examination of the cell type and enzymatic activities of these purified isolates showed significant correlations with the amylolytic and lipolytic activities and the gram stain (see Table 2). Compared with 20 randomly selected isolates (no Kepone), 75% of the Kepone-tolerant isolates displayed amylolytic and lipolytic activities, whereas only 55% of the nontolerant isolates showed these activities. Ninety percent of the Kepone-tolerant isolates were gram-negative compared to 55% of the isolates from Zobell's marine agar (no Kepone). There was no significant difference between the two groups of isolates when compared with respect to the other characteristics tested.

After pure cultures of heterotrophic bacteria have been isolated, their individual growth responses can be scrutinized in relation to toxicant effects. These studies provide information on minimal toxicant concentration and on the range of sensitivity of various isolates. For example, to determine the toxicity of Kepone under different physiological conditions, growth studies were conducted with a rich, complex medium (yeast extract and proteins) and a defined medium (mineral salts and a single carbon source). The bacteria were isolated from estuarine environments in Pensacola, Florida. Isolate 32K, a gram-negative, rod-shaped bacterium, was not inhibited by Kepone at any concentration in complex media (Fig. 3). However, in a defined medium, the growth rate decreased as Kepone concentration increased.

Table 6 Effects of Diuron on the Population of Bacteria that Grow on Selective Culture Media[a]

	Number of colonies			
	Lake water[b]		Laboratory diuron test[c]	
Culture medium	Keowee[d]	Hartwell[d]	Keowee	Hartwell
Plate count	1×10^5	5×10^6	2×10^4	5×10^4
Protein	2×10^3	2×10^5	2×10^3	2×10^4
Starch	1×10^3	1×10^4	0	1×10^3
Lipid	0	3×10^4	0	2×10^4
Glucose salts	3×10^3	2×10^4	5×10^3	2×10^4

[a] From Guthrie et al. (1974).
[b] Counts shown are initial counts.
[c] Counts shown are final counts after test period.
[d] Lakes Keowee and Hartwell are located in South Carolina.

Similar growth studies performed on cultures of two gram-positive cocci (49K and 19K) which grew auxotrophically only in nutrient broth, involved different inoculum sizes (Fig. 4). A 4-fold reduction in growth was observed for the higher inoculum and a 20-fold one for the lower inoculum when they were exposed to 2.0 mg/l Kepone. Apparently, lysis or clumping of cells was responsible for the decline in optical density (growth) with the lower inoculum. In comparison, under the same conditions the growth of isolate 19K (Fig. 4) was depressed (28% optical density decrease) at a much smaller inoculum size, suggesting that 19K was more tolerant to Kepone. This is not surprising, considering that the organism was originally isolated from a toxic waste holding pond that receives a considerable loading of chlorinated hydrocarbons.

A unique autotrophic index was proposed by Matthews et al. (1980) as a possible test of toxic effects on microorganisms. Their approach involves analyzing the changes in the ratio of autotrophic to heterotrophic components of a stream microbial community. They measured microbial

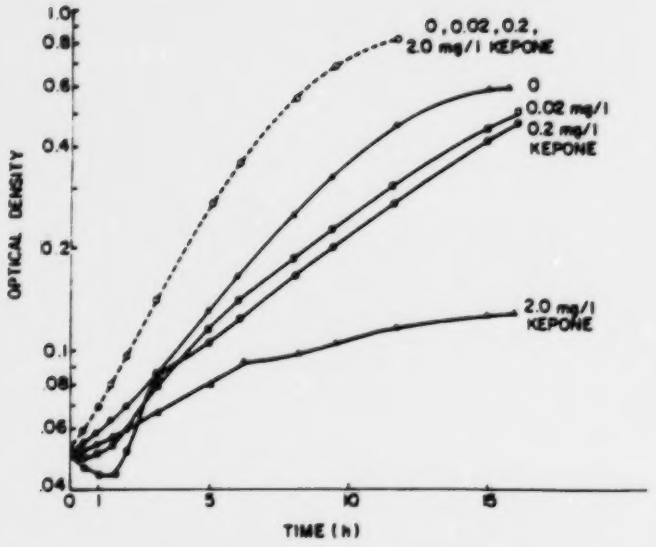

Figure 3 Growth response of isolate 32K on complex and defined media in the presence of Kepone. Controls contain amounts of acetone carrier equivalent to those used in treated cultures. (——) MSB/0.4% succinate medium (defined); (- - - -), Zobell's marine broth (complex) at 1.5% NaCl. *[From Mahaffey et al. (1982).]*

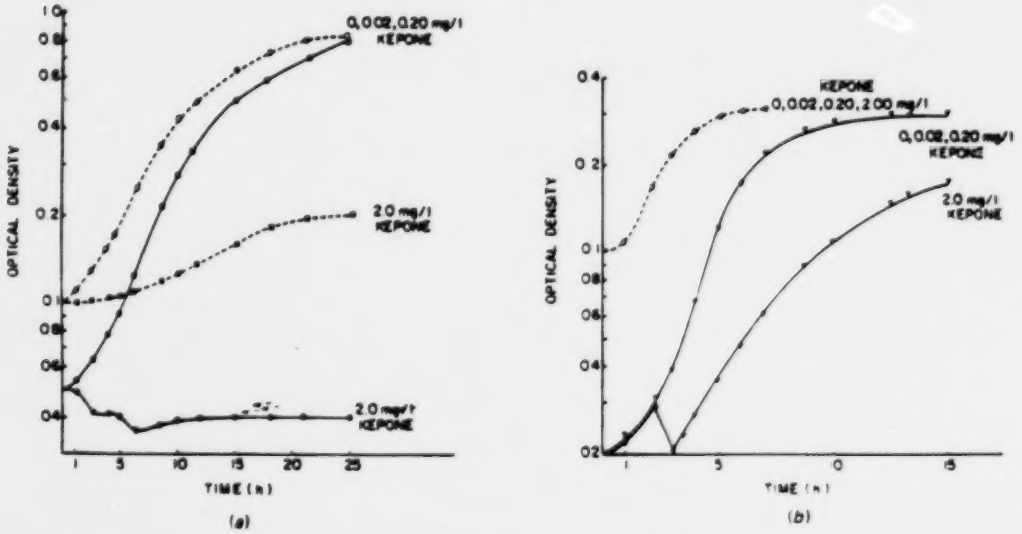

Figure 4 Effect of inoculum size on growth of isolates 49K and 19K in the presence of Kepone in marine broth. (*a*) Isolate 49K; initial optical densities were (——) 0.05 and (- - - -) 0.1. (*b*) Isolate 19K; initial optical densities were (——) 0.02 and (- - - -) 0.1. *[From Mahaffey et al. (1982).]*

biomass (ATP method) and chlorophyll *a* concentration under stressed conditions and then related these data to changes in the structure and function of the stream macroinvertebrate community. They hypothesized that any stress would promote a change in the heterotrophic/autotrophic balance as a result of the growth of tolerant species.

Data were collected from a small stream that received chlorinated sewage effluent near its headwaters and from a relatively unpolluted control stream. Polyurethane sponge substrates were suspended in the streams for 3, 7, and 10 d of colonization. At each sampling period, the microbial community was squeezed from the sponges, and the ATP and chlorophyll *a* content were determined. Microinvertebrates were sampled at the same time, using a box or Surber sampler, and classified into specific functional groups based on feeding habits.

The autotrophic index (AI)—the ratio of microorganism biomass (ATP) to chlorophyll concentration—was examined to determine whether the heterotrophic/autotrophic balance in the streams had been upset. Figure 5*a* is a plot of log AI versus percent of grazers (zooplankton that fed on bacteria and algae). As the AI value increased (i.e., the heterotrophic component increased relative to the autotrophic component) the change in invertebrate community structure was shown by a decreased grazer population. In another example (Fig. 5*b*), the AI increased substantially and the invertebrate population diversity actually decreased. Thus, it appears that the balance between autotrophy and heterotrophy in the stream can be upset. Since both ATP and chlorophyll *a* are relatively simple measurements of standing biomass, and the data show definite relationships to traditional taxonomic responses to stress, this method of monitoring microbial communities could be useful in assessments of toxic effects on microorganisms.

Toward a Standard Bacterial Toxicity Test

Laboratory toxicity tests with fish, invertebrates, or algae are usually single-species tests in which the toxicity of a chemical is measured through mortality, decreased growth rate, and lowered reproductive capacity. These tests have been highly standardized and applied to a select group of organisms. Similar tests for bacteria have not been extensively developed. However, numerous studies have been performed with pure (axenic) cultures of bacteria, and certain types could be used for standard toxicity measurements. If a test organism is selected, there are several possible measurements of microbial activity and microbial growth that could be used in a standard test. Cell growth rates and oxygen uptake look promising for bacterial toxicity tests.

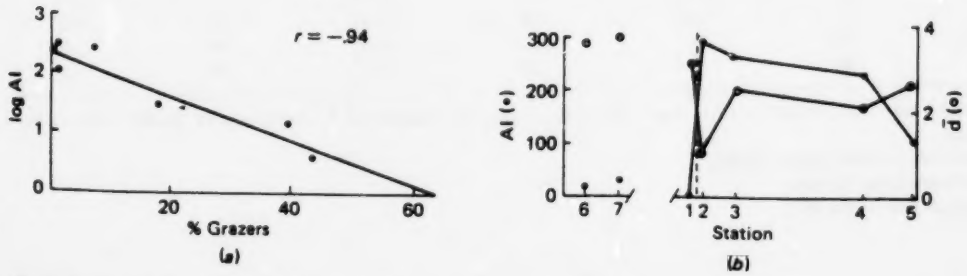

Figure 5 Use of the autotrophic index AI (ratio of microbial biomass to chlorophyll concentration) to assess pollution stress. (*a*) Linearity of the index with n[illegible] of grazer organisms within a total invertebrate population. (*b*) Change in the index and in n[illegible]nvertebrate diversity $\bar{d}(0)$ at several river stations. *[From Matthews et al. (1980).]*

Growth rate constants of pure cultures of bacteria can be readily obtained. The organism can be inoculated into a compatible medium in a shake flask. Growth can be monitored by measuring optical density of the cell suspensions, and the values can be converted to dry weight for each organism tested. By plotting the natural logarithm (ln) of the cell weight against time and multiplying the slope by 7.303, a growth rate constant can be obtained. If the same experiment is performed in the presence of different concentrations of the toxicant, the investigator can determine inhibition or stimulation of the organism's growth.

Breazeale and Camper (1972) used pure cultures and changes in growth rate constants to measure the toxic effects of several selected herbicides (Table 7). The three test organisms were *Erwinia*, *Pseudomonas*, and *Bacillus*, all common inhabitants of terrestrial environments. Several of the chemicals tested inhibited growth (e.g., diquat completely inhibited growth of *Bacillus* sp.); others stimulated growth (nitralin versus *Pseudomonas*); and others had no effect (picloram versus *Erwinia*). Each organism showed a completely different spectrum of sensitivity to the toxicants. For particular chemicals, all of the organisms showed initial growth inhibition, but then apparently became acclimated and grew. This is a simple, straightforward pure culture toxicity test which can be considered as an acute toxicity test. Growth can easily be measured using increases in turbidity, in approximately 2–4 d. A test for the ability to adapt to better growth in the presence of the herbicide after a long incubation period could be considered a chronic test.

Trevors and Basaraba (1980) and Mahaffey et al. (1982) developed a pure culture toxicity test involving a respirometric or oxygen uptake method. In the former study, glucose was the principal carbon and energy source for a culture of *Pseudomonas fluorescens*. Cells grown in a compatible medium and washed three times in phosphate buffer provided a resting cell culture which was placed in a Warburg respirometer. Oxygen uptake measurements were made before and after the addition of glucose and in the presence and absence of a toxicant (Fig. 6). At relatively high concentrations (10 μg/ml) of benzoquinone and hydroquinone, oxygen uptake was significantly inhibited, even below endogenous levels.

Mahaffey et al. (1982) performed similar experiments to examine the effects of Kepone.

Table 7 Growth Responses[a] of *Erwinia carotovora*, *Pseudomonas fluorescens*, and *Bacillus* sp. to Selected Herbicides[b]

	Erwinia carotovora		*Pseudomonas fluorescens*		*Bacillus* sp.	
Herbicide	Minimal response concentration (μg/ml)	Response (%)	Minimal response concentration (μg/ml)	Response (%)	Minimal response concentration (μg/ml)	Response (%)
Diquat	25	40.8[c]	50	0	25	100[c]
Loxynil	25	71.0[c]	50	0	10	29.0[d]
Paraquat	50	25.5[c]	25	43.7[c]	5	100[c]
PCP	10	29.6[d]	25	65.8[d]	5	100[c]
Picloram	50	0	50	28.8[d]	50	0
Nitralin	50	0	25	22.0[d]	50	0

[a] All responses to the herbicides listed were inhibitory, except the response of *P. fluorescens* to nitralin, which was stimulatory.

[b] From Breazeal and Camper (1972).

[c] Significant at the 1% level.

[d] Significant at the 5% level.

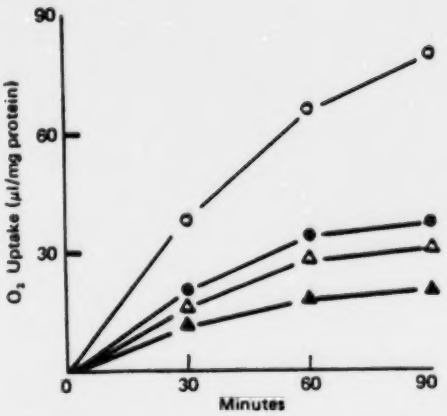

Figure 6 Inhibition of oxygen uptake by resting cells in the presence of (▲) benzoquinone and (△) hydroquinone at 10 µg/ml; (○) control and (●) endogenous condition. *[From Trevors and Basaraba (1980).]*

Cultures grown in a minimal salts medium were placed in a Gilford Oxygraph respirometer, and oxygen uptake rates were measured with an oxygen probe. At constant uptake, Kepone was added and its effect on the oxygen uptake was recorded continuously (Fig. 7). Respiration was reduced at about 20 mg/l Kepone. Kepone was considerably more toxic than pentachlorophenol. With one isolate, Kepone actually stimulated the production of oxygen (Fig. 8). The reason for this response is not yet known.

Respirometry provides data on metabolic processes that may not be observed in growth studies. The test is sensitive and short (about 1-2 h) and is easily standardized. It has been used for years to study intermediary metabolism in bacteria, and hence toxic effects can be related to the physiology of the organism. The procedure can be used to examine oxygen uptake by cell-free extracts of bacteria. Mahaffey et al. (1982) showed that Kepone toxicity may be related to the oxidative metabolism of microorganisms. The effects of Kepone on electron transport activity were studied by measuring the inhibition of NADH oxidase and succinooxidase activities in the particulate fraction of cell extracts or the electron transport particle (ETP). To obtain the ETP preparation, cells grown for 18 h in succinate medium, harvested by centrifugation, washed, and resuspended in buffer were disrupted by sonication. Cell debris was removed by centrifugation and the supernatant centrifuged at 150,000 × *g* for 1 h at 2°C. The final pellet was completely homogenized in cold buffer. Succinooxidase activity in the ETP was assayed by measuring

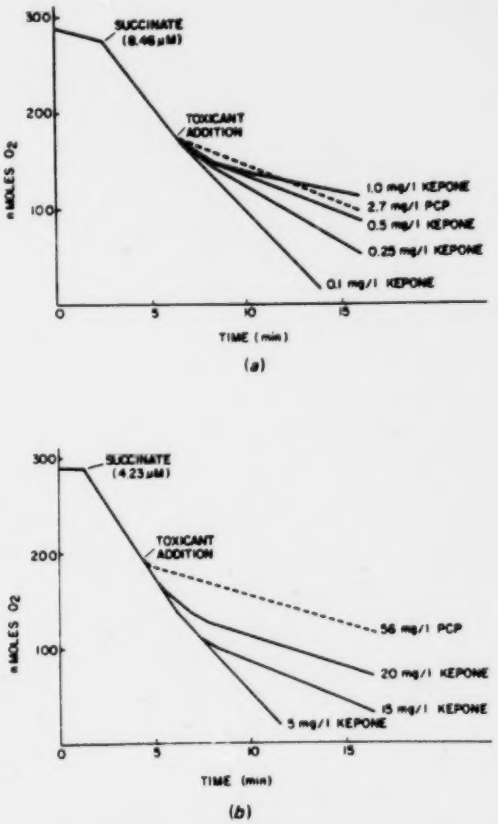

Figure 7 Oxygen uptake activity by whole cells. (*a*) Isolate 32 K (140 µg protein). (*b*) Isolate 28K (180 µg protein). Ten microliters of Kepone or PCP (reference toxicant), in dimethyl formamide carrier, was added to each reaction mixture after a constant rate of O_2 uptake was observed. *[From Mahaffey et al. (1982).]*

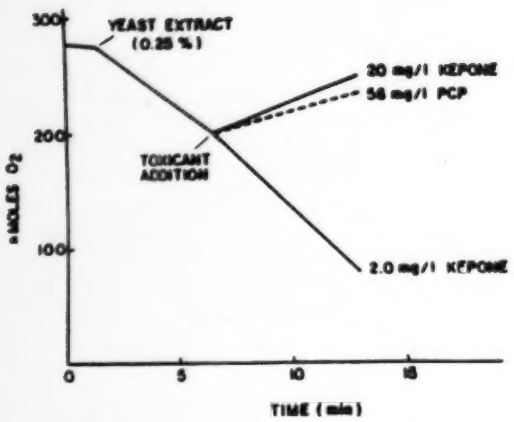

Figure 8 Oxygen evolution by whole cells of isolate 49 (200 µl protein) in the presence of high concentrations of Kepone and PCP. Ten microliters of toxicant in DMF carrier added to reaction mixture after a constant rate of O_2 uptake was observed. DMF alone had no effect. *[From Mahaffey et al. (1982).]*

oxygen uptake. A polarographic procedure was used similarly for NADH oxidase assays, and it was found that the NADH oxidase activity was inhibited 51% by 0.6 mg/l Kepone while succinoxidase activity was inhibited 25%. The maximum reduction in NADH oxidase activity (62%) was obtained with 1.2 mg/l Kepone, while succinoxidase activity was inhibited 50% in the presence of 6 mg/l Kepone. The lower activity of succinoxidase relative to NADH oxidase is characteristic of the ETP.

The lack of Kepone toxicity in anaerobic plate assays and the marked inhibition of oxygen uptake with several isolates indicated a possible involvement with electron transport and respiration. Widdus et al. (1971) and Trudgill et al. (1971) showed that the inhibition of NADH oxidase activity in *Bacillus subtilis* by chlordane occurred indirectly through apparent disruption of membrane-mediated electron transport. If similar mechanisms function during Kepone poisoning, a different effect would be expected from anaerobic and aerobic conditions. Trudgill et al. (1971) did not report on anaerobic growth effects but did show that a *Streptococcus* species (which would have minimal cytochrome-mediated electron transport activity) was significantly more resistant to chlordane. These studies indicate that Kepone probably exerts a direct effect on bacterial membrane integrity and function. Mahaffey et al. (1982) studied the membrane-bound oxidases of the ETP from isolate 32K and showed that NADH oxidase and succinooxidase activities are inhibited by Kepone, indicating disruption of electron transport. Thus disruption of membrane-mediated electron transport may result in a transient imbalance in the oxidation-reduction potential of the cell. These methods can be employed to gain more information about the mechanism of toxicity to bacteria.

EFFECT OF TOXICANTS ON MICROBIAL ACTIVITIES

In toxicity studies with higher organisms, researchers have been examining mortality and growth as possible indicators of stress in pollution situations. Yet more subtle effects may be detectable if functional or physiological attributes are examined. Respiration (O_2 uptake or CO_2 production), gill ventilation rates, enzyme levels and activities in blood, avoidance, feeding rates, and assimilation could be more sensitive to a toxicant and could replace some of the tests involving growth and mortality.

A functional approach to the study of aquatic microbial toxicology has a number of appealing aspects. A large bank of physiological tests with bacteria can serve as alternative toxicity testing techniques. These physiological tests are considerably more developed and refined for microorganisms than they are for higher organisms. They include measures of general heterotrophic activity, geochemical cycling rates, and simple mineralization rates of organic materials. Geochemical cycling rates of sulfur, nitrogen, and phosphorus are particularly important because they are characteristic of a rather select group of

microorganisms, and the disruption of these processes by a toxicant can have very obvious and dangerous environmental consequences. The oxidation of organic material under anaerobic conditions to methane and the decomposition of bulk organic matter (plant and animal litter) are good functional measures with which to gauge the harmful effects of a toxicant. The assessment consists of measuring substrate disappearance or the production of some product. The advantages and disadvantages of a variety of functional measures related to bacterial activities are discussed in the following sections.

Heterotrophic Activity

Numerous studies to characterize the metabolic activities of microorganisms in natural environments have concentrated on the rates at which bacteria transport solutes into the cell (Wright, 1978) and the rates at which bacteria mineralize a particular chemical to carbon dioxide (Harrison et al., 1971). The success of the kinetic approach for studying the natural activities of bacterial populations has led to a multifaceted application of the techniques to study aquatic microbial ecology. In heterotrophic activity studies, the metabolism of a radiolabeled organic substance (e.g., a sugar, amino acid, or organic acid) is measured with natural assemblages of microorganisms. It is assumed that if the concentration of the added substrate is kept low, it will be metabolized by the bacterial populations in a water sample at a rate similar to that actually occurring in the environment. The assay for metabolism is either uptake of the radioactive material into the cell or mineralization to carbon dioxide. The metabolic rates can be described by the Monod growth equation which relates bacterial growth to the concentration of a growth-limiting component, such as the carbon and energy source. This is an important relationship because it seems to apply to mixed microbial populations in which not all bacteria are active or active to the same extent. The Monod equation can be used to estimate rates at which certain carbon compounds are metabolized by bacteria (turnover rates), the maximum growth rate of a microbial population, and the affinity of that population for the carbon source. Thus, it is a very useful method for measuring quantitatively the metabolic activities of natural heterotrophic populations. For further details of the technique and examples of the kinetic information derived in various types of studies, the reader is referred to Colwell and Morita (1974).

Hodson et al. (1977) illustrate the application of the technique to a specific toxicological problem: the potential effects of a crude oil spill on natural populations of marine bacteria. The experiments were part of the CEPEX program (Menzel and Case, 1977). Two large (68 m^3) polyethylene bags were used to enclose large water masses. Several crude oils were added to one bag to give a final hydrocarbon concentration of 10 μg/l (ppb). The other bag served as the unexposed control. Seawater was removed from the control and oil-treated bags with 6-l Niskin bottles and the water samples were passed consecutively through 183- and 35-μm Nitex mesh filters and finally through 1-μm (pore diameter) Nucleopore filters. This effectively separated the photosynthetic organisms from the bacteria and thus permitted a specific study of the oil-induced changes in heterotrophic activity of natural bacterial populations.

To measure the effect of various concentrations of four types of crude oil on glucose assimilation into the cell, 25-ml filtered water samples (in triplicate) were placed in sterile bottles containing 0 to 3 ml oil-saturated aqueous solution. The bottles were capped and incubated in the dark at the temperature of the water sample at collection for 4 h, after which D-[^{14}C]glucose (about 10^5 cpm) was added to the bottles and incubation continued for 2 h. In many studies, serum bottles are used so that the radioactive glucose could be added with a syringe. (Glucose is one of several substrates that can be used; amino acids, hydrocarbons, glycolic acid, organic acids, and urea have all been successfully tested.) At the end of 2 h the water is filtered through a 0.22-μm

membrane filter and washed in sterile seawater. The amount of radioactivity remaining on the filter gives the amount of glucose assimilated by the cells. It is assumed either that none of the substrate is lost by mineralization to CO_2 (due to the short incubation period) or that any loss is compensated by actual measurements (see below).

Two types of kinetic approaches (not described in the Hodson paper) have been used in assimilation experiments to ensure that the added substrate does not bias the assimilation rate (Wright, 1978). In one, a concentration of substrate below the *in situ* concentration (generally 50–100 μg/l for aquatic environments) was used and therefore did not influence uptake rates by the bacteria. In the second, concentrations higher than natural levels were used, but several concentrations were tested. By plotting the rate of uptake (fraction assimilated per minute) against the reciprocal of the added substrate concentration, a straight line can usually be obtained. Extrapolation to zero added substrate gives the assimilation rate at natural substrate concentrations. Hodson et al. (1977) chose a concentration based on the workability of the radioactivity levels. The assumption is made that as long as the same concentration of substrate is used, sample-to-sample, the responses of bacterial populations to the substrate will be the same and relative effects of a toxicant can be easily observed.

Hodson et al. (1977) studied the effects of oil on mineralization (bacterial conversion of glucose to CO_2) in sealed (with rubber septa) culture tubes equipped with a hanging center-well to hold CO_2 trapping agent (Fig. 9). The trapping agent can be either a commercial preparation or a chemical such as phenylethylamine or ethanolamine. A small piece of fluted filter paper is usually placed in the cup to increase surface area and trapping efficiency. The tubes are inoculated and incubated as in the assimilation experiments, but at the end of the 2-h incubation, acid is added to the tube with a syringe by inserting a needle through the rubber septum to avoid opening the vessel. The acid stops the reaction and releases any dissolved radioactive CO_2 from the water. The trapping

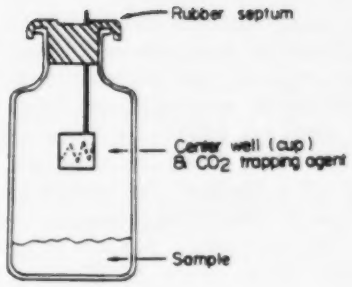

Figure 9 Reaction bottle used to study heterotrophic activity in natural microbial populations.

agent is added to the cup with a second syringe. After approximately one-half h all the CO_2 in the system is trapped. The bottles are opened, and the filter paper is placed directly into scintillation fluid for radioactivity counting. Since the radioactivity in the trapping agent is due entirely to radiolabeled CO_2, the extent of mineralization can be quantitated. This same procedure can be used to study mineralization of substrates in sediment-water slurries (Harrison et al., 1971).

An example of how the toxic effects of crude oils can be assessed in this way is shown in Fig. 10. The oils inhibited glucose assimilation, the effect increasing with increasing concentration of oil. Oil concentrations in excess of 300 μg/l were required to produce inhibition, and the oils showed different degrees of toxicity. Bunker C fuel oil (80 μg/l) stimulated assimilation, probably as a result of the presence of growth-promoting substances. Similar results were observed with mineralization. The authors concluded that a 30-d exposure to crude oil in the enclosed water sample did not result in acclimation of the microbial population to the oil. This lack of acclimation could have been due to the exposure time, which was short compared to the time the bacteria were exposed in their natural substrate, or to the low oil concentration (~10 μg/l). Nevertheless, measurement of heterotrophic activity appears to be useful for measuring the toxic effects of chemicals to microbial populations.

Orndorff and Colwell (1980) used heterotrophic activity in toxic effects studies in a slightly different manner. Since two water samples with different heterotrophic activities may result from different concentrations of bacteria in the samples, measurements of heterotrophic activity were coupled with direct counting methods. By reporting a rate per cell, such as V_{max} (maximum growth rate) or uptake rate, a specific activity index is obtained. Wright (1978) counted bacteria by the acridine orange epifluorescence (ADOC) method and found habitat differences along a river and into an estuary. Orndorff and Colwell (1980) used the ADOC technique to determine the effects of Kepone on microbial populations in samples of James River (Virginia) water. They modified the method according to the procedure of Kogure et al. (1979) to allow separation of live and dead cells (see section on Enumeration of Bacteria in Aquatic Environments). They based their specific activity measurement on the total uptake and mineralization of ^{14}C-labeled amino acids (MA) as a function of the viable ADOC number (DVC), taken as either Kepone-resistant or Kepone-sensitive bacteria.

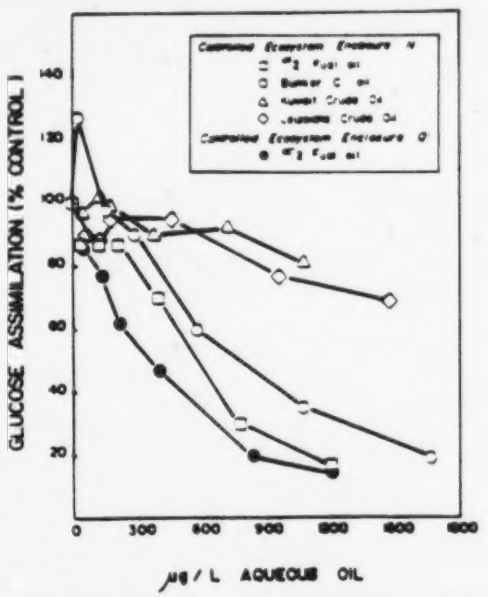

Figure 10 Assimilation of D-[U-^{14}C] glucose (as percent of control value) by 1-µm filterable population from "controlled ecosystem enclosure N" in the presence of increasing concentrations of No. 2 fuel oil, bunker C oil, Kuwait crude oil, and Louisiana crude oil and by 1-µm filtrate from "controlled ecosystem enclosure O" in the presence of increasing concentrations of No. 2 fuel oil. Incubations were done in triplicate. In all cases, values for the replicates varied by less than 2% of the mean. *[From Hodson et al. (1977).]*

The MA/DVC index based on Kepone-resistant bacteria (those able to grow in the presence of Kepone at ≥0.1 mg/l) was not much different from that based on sensitive cells (Table 8). This means that bacteria resistant to Kepone have about the same metabolic activity as sensitive cells (at concentrations of Kepone below 0.1 mg/l), and it suggests that bacteria which predominate as a result of a toxicant stress will be able to carry on the same metabolic activity as those which were dominant before the stress. Thus microbial populations appear to be very adaptable to a range of perturbations caused by toxic chemicals. In similar studies, Goulder et al. (1980) (see section on Growth of Heterotrophic Microorganisms) used a specific activity index of V_{max} of glucose uptake per cell; the same relationship to toxicant stress was observed. In their studies V_{max} per cell was negatively correlated with copper concentration, whereas percent mineralization was not correlated. Thus specific activity indices appear to be an excellent measure of toxicity to actively metabolizing microbial populations in natural water samples. They are also useful as potential indicators of pollution stress.

Geochemical Cycling: Nitrogen

Cycling of inorganic nutrients involves the chemical and biological transformation of a variety of nitrogen, sulfur, and phosphorus compounds. Since the principles behind these transformations are similar, only nitrogen cycling will be discussed in relation to toxicity testing. Information on the other inorganic compounds can be found in the literature cited.

Mineralization of organic matter by bacteria

Table 8 Effect of Kepone on Respiration and Incorporation of ^{14}C-labeled L-Amino Acids and Metabolic Activity of Chesapeake Bay Microorganisms[a]

	Eastern Bay				Colgate Creek			
			MA				MA	
Kepone concentration (mg/l)	Respiration (%)[b]	Incorporation (%)[b]	Kepone DVC[c] ($\times 10^{-5}$)	DVC[c] ($\times 10$)	Respiration (%)	Incorporation (%)	Kepone DVC[c] ($\times 10^{-5}$)	DVC[c] ($\times 10$)
0.01	100	100	7.64[d]	5.93[e]	62.2	43.3	4.00[f]	2.09[g]
0.05	–	–	–	–	25.7	24.9	2.77	1.10
0.1	34.5	53.1	6.63	2.86	–	–	–	–
0.2	17.2	11.3	5.92	0.76	18.8	17.3	1.20	0.78
1.0	14.9	9.6	2.40	0.65	18.1	16.5	1.20	0.58
10.0	9.6	4.7	2.40	0.28	16.5	15.1	1.20	0.41

[a] From Orndorff and Colwell (1980).
[b] Percent of control, i.e., without added Kepone, after incubation for 2 h.
[c] Metabolic activity/cell = [(respiration + incorporation)/ml water]/[DVC/ml water or Kepone DVC/ml water].
[d] Eastern Bay control, MA/Kepone DVC = 8.50×10^{-1}.
[e] Eastern Bay control, MA/DVC = 6.06×10.
[f] Colgate Creek control, MA/Kepone DVC = 6.10×10^{-1}.
[g] Colgate Creek control, MA/DVC = 4.40×10^{-1}.

results in the production of ammonia (i.e., conversion of organically bound nitrogen to free nitrogen). Although ammonia is used by plants, nitrate is the principal inorganic nutrient used by rooted plants and algae since it is the form of nitrogen most readily assimilated. Much of the ammonia must therefore be converted to nitrate through nitrification. This conversion is mediated almost entirely by two groups of bacteria: *Nitrosomonas* sp., which oxidizes the ammonia to nitrite, and *Nitrobacter* sp., which oxidizes the nitrite to nitrate. The nitrifying bacteria are chemolithotrophs, which utilize the energy derived from nitrification to assimilate CO_2; consequently, they are slow growers. This is important in relation to toxicity studies. If these bacteria are killed or disturbed the population recovery could be quite slow; this could disrupt the nitrogen cycle significantly and have long-lasting effects on the productivity of an ecosystem. In contrast, organisms that release ammonia from organic materials (ammonification) quickly return to the soil and resume functioning rapidly after exposure to a toxicant. If the activity of the nitrifers is slowed or stopped for weeks to months, depending on the chemical and environmental conditions, the accumulation of ammonia from decaying organic matter might occur and this could generally foul the environment.

Because of this potential effect, investigators have tested many chemicals for effects on nitrification, particularly in terrestrial environments. Test methods have been developed to the extent that nitrification could be used as a routine toxicity test.

Nitrification is measured as follows. Samples from the environment are supplemented with ammonia (usually as ammonium chloride or ammonium sulfate), and the rate and extent of nitrite and nitrate production are measured in the presence and absence of a toxicant. For example, Jones and Hood (1980) examined the effects of seven thiophosphorus pesticides on ammonia oxidation. Ammonia-oxidizing bacterial populations were obtained by selective enrichment from sediment cores taken from an estuary. Subsamples of the sediment were removed from the cores and placed in a minimal salts medium containing

ammonium chloride as the sole nitrogen source (selective for ammonia-oxidizing bacteria). Production of nitrite was determined by a standard colorimetric method in the presence and absence of the pesticides. The response of the natural populations of nitrifers to several pesticides at 10 mg/l is shown in Fig. 11. Control sediments produced nitrite in about 6 d of incubation and nitrite concentrations continued to rise until d 14. The pesticides all inhibited nitrification to some extent; fonofos and guthion (10 μg/l) were the most inhibitory of the pesticides tested, and methyl parathion and parathion were transiently inhibitory.

The principal degradation products were also examined for toxicity. Aminophenol, for example, was much more toxic (producing an effect at lower concentrations) than its parent compound, methyl parathion. Several pure cultures of bacteria capable of oxidizing ammonia were generally less sensitive to the pesticides than the mixed population from which they were originally isolated.

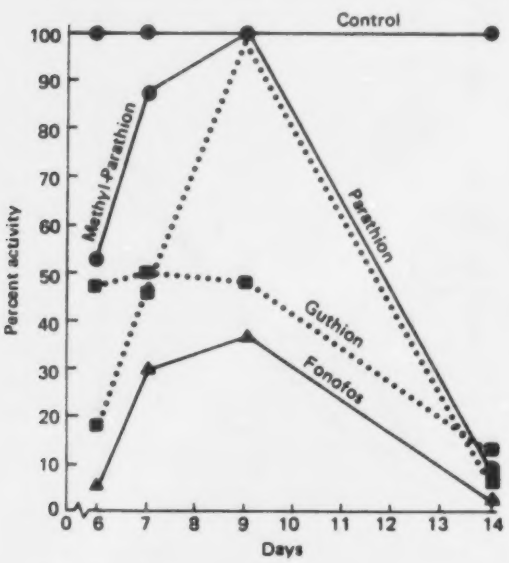

Figure 11 Effect of four thiophosphorus pesticides on ammonium oxidation by natural estuarine populations of nitrifiers. Activity is expressed as percent of control levels of nitrite released. *[From Jones and Hood (1980).]*

Studies of toxic effects of chemicals on nitrification rates can be difficult to interpret because of nitrate reduction, which commonly involves the conversion of nitrate to nitrogen gas (denitrification). Denitrification requires anaerobic conditions. In samples containing sediment, reducing conditions are likely to develop and stimulate denitrification. Such reducing conditions can also exist in microniches in apparently aerobic sediments, and denitrification can occur simultaneously with nitrification.

To separate these processes for a toxicological study, a test system for nitrification can be heavily aerated to diminish denitrification. The extent of denitrification can also be estimated by difference, using the chemical N-Serve to inhibit nitrification specifically (Billen, 1975). An alternative is to use the ^{15}N isotope dilution technique (Koike and Hattori, 1978). Sediment samples are placed in bottles and amended with sterile water, ^{15}N-labeled nitrate, and unlabeled ammonia. After complete mixing and incubation for various time periods, the contents are filtered through glass fiber filters, and the filtrate is saved for analysis. The analysis consists of converting all the nitrate in the sample to nitrite (by cadmium-copper reduction) and then converting the nitrite to nitrogen gas. The ratio of $^{29}N_2$ (formed from the added ^{15}N nitrate) to $^{28}N_2$ (from nitrification of unlabeled ammonia) is determined by mass spectrometry. Only nitrification and nitrate reduction are responsible for the nitrate change in sediments, and their rates can be calculated from the following equations:

$$N_2 - X_1 = Z - Y$$

$$N_2X_2 - N_1X_1 = Z\bar{x}Za - Y\bar{x}$$

where Y is the consumption of nitrate by bacterial nitrate reduction, Z is the production of nitrite by nitrification, N and X are the concentration of nitrate and its ^{15}N content, respectively, $\bar{x}$ and $\bar{x}Za$ are the average ^{15}N contents of nitrate and ammonia between times t_1 and t_2, respectively.

The type of data that can be developed by this technique (rates of nitrate reduction and nitrification) is shown in Fig. 12. The technique is reliable and can be easily applied to toxicological studies. Both nitrification and denitrification can be examined simultaneously, providing a much better indication of the toxicological effect of a chemical on the nitrogen cycle in natural systems.

A wealth of information exists on nitrogen fixation (the formation of nitrogen compounds from free N_2) in aquatic and terrestrial environments. This could provide another good criterion for the effect of a toxicant on a microbial system. Nitrogen fixation is carried out by several groups of microorganisms. In terrestrial environments, fixation of nitrogen by *Rhizobium* in symbiotic association with plant roots accounts for most of the nitrogen converted into organic compounds. A number of free-living soil bacteria, such as *Azotobacter* and *Beijerinckia*, also fix nitrogen, but at a much lower rate. In aquatic environments, the major nitrogen fixers are the cyanobacteria *Nostoc* and *Anabaena*. These organisms are generally free-living and it is relatively easy to measure their nitrogen-fixing capability. Aquatic studies have shown that *in situ* measurements can be obtained with belljar-like systems (Bohool and Wiebe, 1978) or sealed cores (Herbert, 1975). A variety of methods are also available for studies with laboratory systems (Lee and Watanabe, 1977).

Studies of rates of nitrogen fixation have been facilitated by the development of the acetylene reduction method. This method is based on the fact that the enzyme nitrogenase, which is re-

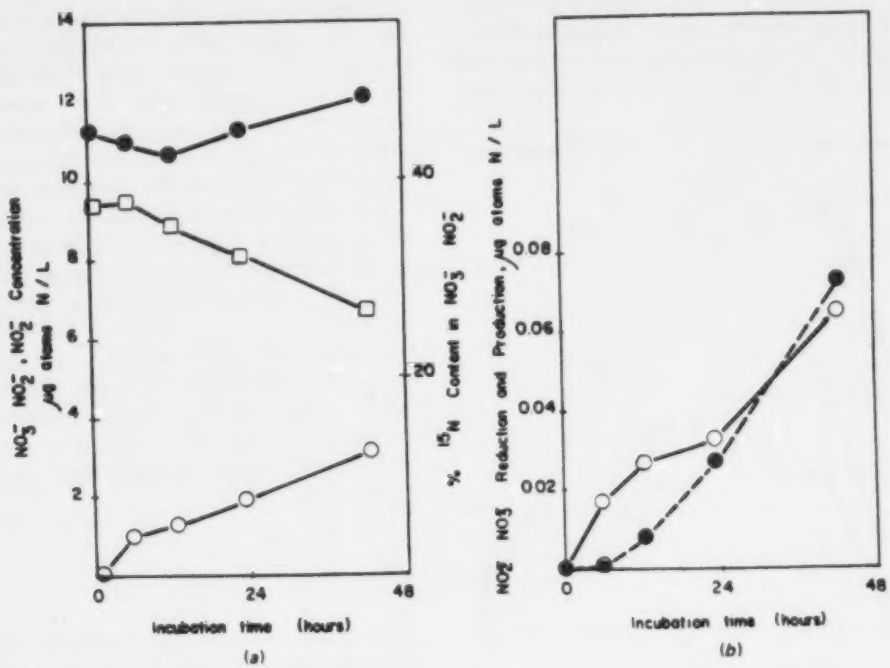

Figure 12 Nitrate reduction and nitrification in sandy sediments from Odawa Bay (November 20, 1976). (*a*) Changes with time in (○) nitrate and nitrite, (●) nitrite, and (□) ^{15}N content in nitrate and nitrite. (*b*) Nitrate (○) reduction and (●) production were calculated with equations in the text. *[From Koike and Hattori (1978).]*

sponsible for reducing N_2 to ammonia, also reduces acetylene. In this technique, vessels are purged of nitrogen and the atmosphere is replaced with helium and acetylene. Conversion of the acetylene (which acts as an N_2 analog) to ethylene is measured by flame ionization gas chromatography. The method works well in aquatic test systems. The results are often difficult to interpret in sediment-containing systems, where the measured rates may be more dependent on the diffusion of acetylene and ethylene to and from the active sediment sites (Lee and Wontanabel, 1977) than on N_2 fixation. Herbert (1975) found large differences between *in situ* and laboratory measurements, presumably due to gas diffusion limitations. Attempts to rectify this problem invariably result in disrupted sediment-water interfaces and uptake rates that cannot be unequivocally attributed to the natural system. However, the methods available for studying nitrogen fixation in aquatic environments are certainly adequate to provide information on relative toxicity. Although there have been numerous studies of nitrogen fixation in aquatic systems, few toxicological studies have been performed. To illustrate how nitrogen fixation can be studied in relation to toxicant input, some recent experiments have been made in soil systems whose results could be applied to aquatic systems.

Blue-green algae significantly influence the nitrogen economy of aquatic environments. DaSilva et al. (1975) used acetylene reduction to test six pesticides for their effect on nitrogen fixation by eight axenic cultures of a symbiotic blue-green alga. Mixed results were obtained; with some pesticides an initial depression in nitrogen-fixing activity of the algal species was followed by a dramatic increase in activity (Fig. 13). With others, such as monuron or MCPA (4-chloro-2-methyl-phenoxyacetic acid), significant inhibition was apparent throughout the exposure period. Some species were affected more than others (both stimulation and depression of activity), and in some cases acclimation to the presence of the pesticide occurred after extended incubation.

Charyulu et al. (1980) studied nitrogen fixation in a flooded rice paddy and effects of different concentrations of a benzimidazole pesticide. At the end of each incubation period, subsamples were spread on agar plates containing nitrogen-free medium to enumerate the symbiotic nitrogen-fixing organisms. Mannitol is usually used as the carbon source; great care must be taken to ensure that there is no combined nitrogen from other ingredients of the medium. Other subsamples were placed in tubes under an acetylene atmosphere, and nitrogen fixation was measured. The pesticide generally reduced cell numbers but did not substantially affect nitrogen fixation. This study indicates that an activity measure is better than a population measure because nitrogenase activity depends on the effectiveness of the individuals rather than their numbers. It also shows that this pesticide retarded the normal drop in oxidation-reduction potential for samples of flooded soil. Nitrogen fixation by microorganisms is very sensitive to Eh. The drop in Eh normally would favor the nitrogen-fixing organism *Azospirillum* (a common inhabitant of these soils); a stabilized Eh then would presumably inhibit the nitrogen-fixing potential of the soil.

Decomposition Processes

Bacteria and fungi are primarily responsible for the decomposition of bulk organic matter produced in aquatic environments. Most of this organic matter comes from plant debris. It is either transformed into soluble organic substances and eventually mineralized to carbon dioxide, or transformed into particulate organic carbon, which may become resistant to microbial breakdown. Accumulation of humic acids, pectins, lignins, and chitin (from animal sources) may be the result of the latter degradation processes. Microbial transformations require a wide range of enzymes, including cellulases for plant material, lipases for lipids, amylases for starch, proteases for proteins, amidases for chitin, and oxygenases for lignin. A single bacterial species probably will not have the genetic information for more than one or two of

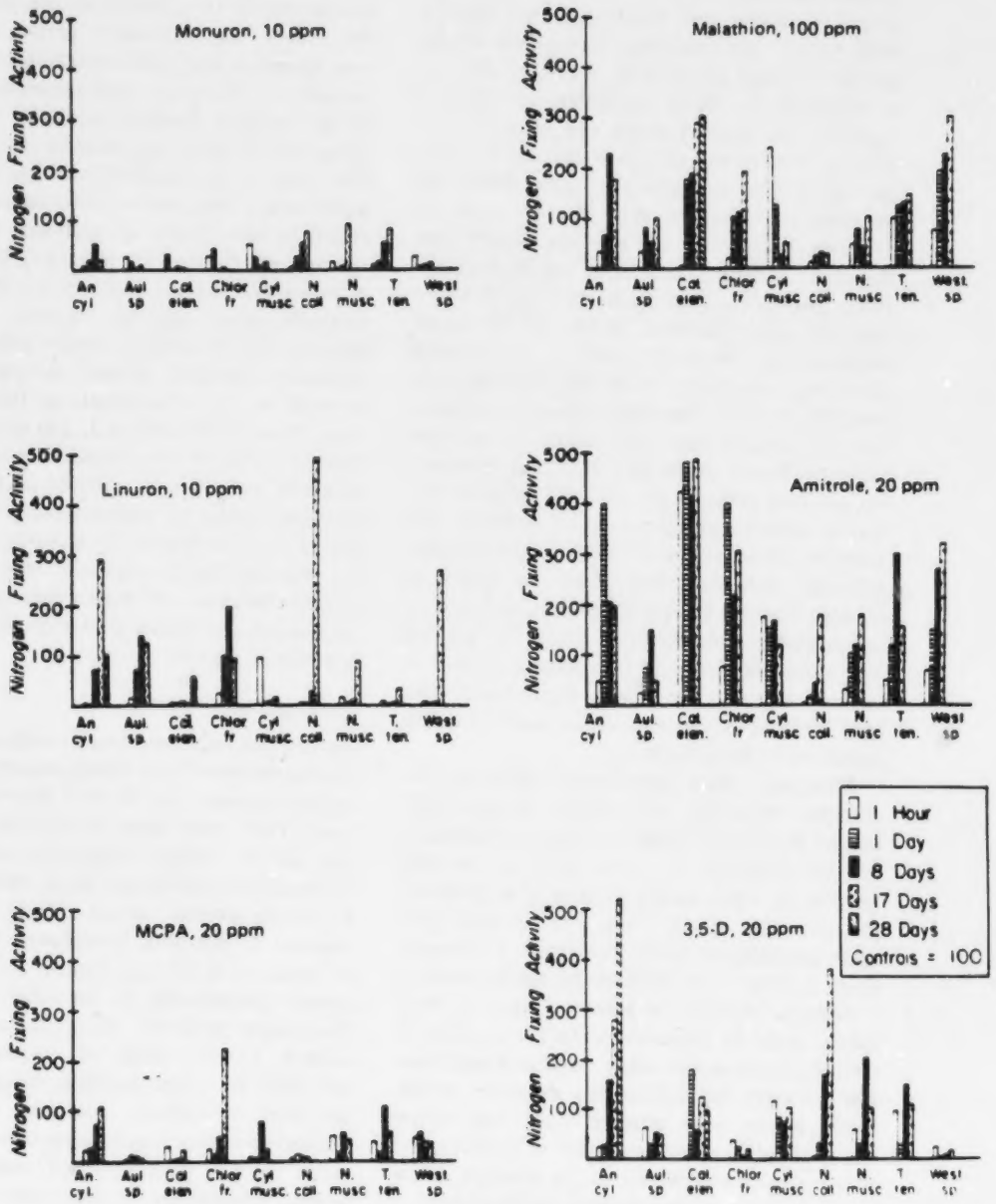

Figure 13 Effect of monuron, linuron, MCPA, malathion, amitrole, and 3,5-D on the nitrogen-fixing ability of *Anabaena cylindrica*, *Aulosira* sp., *Calothrix elenkenii*, *Chlorogloea fritschii*, *Cylindrospermum muscicola*, *Nostoc* sp. from *Collema*, *Nostoc muscorum*, *Tolypothrix tenuis*, and *Westiellopsis* sp. An initial inhibitory effect is more or less followed by gradual recovery. In some cases stimulation may occur. *[From DaSilva et al. (1975).]*

these enzymes. But these activities can be found in a typical interacting population of aquatic bacteria, where commensalistic and mutualistic coordination among bacteria themselves, between bacteria and fungi, and among bacteria and higher plants and animals can be found. A chemical that disrupts one of these enzymatic processes may ultimately alter the metabolic capability of the entire population.

Most streams and rivers have dense vegetation along their banks. When the vegetation dies, it commonly enters the water as leaf litter and serves as the dominant energy source, or an allochthonous input. Animal and microbial components of the stream are largely responsible for decomposing this litter material and releasing available energy. This decomposition process is a commensal relationship between animals and microbes. The animals shred the leaf litter into small chunks with large surface areas which can be colonized and decomposed by the bacteria and fungi. The microorganisms themselves are a nutrient source for grazing invertebrate animals. Thus, if the bacterial and fungal populations were disturbed or inhibited, a drastic change in community structure could occur and secondary productivity could be greatly reduced.

Giesy (1978) examined the toxic effects of cadmium by studying its impact on leaf litter decomposition. Fresh leaf material was placed in stainless steel screen envelopes and the package suspended 10 cm above the sediment surface in a model outdoor stream facility. The stream contained many of the assemblages of macrophytes, periphyton, micro- and macroinvertebrates, and fish typically found in a stream. Separate streams were treated continuously with $CdCl_2$ at 5 and 10 μgl, and changes in the leaf pack were noted. Both concentrations of cadmium reduced leaf litter decomposition, as indicated by changes in dry weight of the leaf pack. Visual observation showed that the exposed leaves (still green and intact after 28 d) were decaying more slowly than the control leaves (brown with only veins and petioles remaining). Pieces of the leaves were also fixed and observed under a scanning electron microscope. The exposed leaves showed much less colonization by bacteria and fungi than the control leaves (Fig. 14).

The apparent inhibition of leaf litter decomposition occurred at cadmium concentrations lower than those needed to inhibit other microorganisms (usually in pure culture) and their activities. It therefore appears that this decomposition process may be very sensitive to toxicants and an early indicator of pollutant stress. This example also shows that a complex mixed natural community may be a better system in which to carry out effects studies than its component parts (pure or axenic cultures).

Anaerobic environments occur in many ecosystems, and the activities of bacteria therein are crucial to the cycling of organic carbon. Methanogenic bacteria and many obligate anaerobic heterotrophic microorganisms have fermentative capacities. The persistence of a toxicant may increase under anaerobic conditions, because fewer organic structures are degraded under anaerobic than under aerobic conditions. A chemical that is also toxic to microbial communities could alter the microbial ecology of anaerobic environments.

There are two basic approaches to studying toxic effects in anaerobic systems. One is to study a specific microbiological process, such as methane production; the other is to study the integrated activities of a large number of organisms and chemical processes. Measurement of the respiratory electron transport system in aquatic sediments is an example of the latter approach.

Respiratory electron transport system (ETS) is a term used in connection with the respiration of organisms in sediments and is measured by the amount of oxygen taken up by a sediment sample. The oxygen demand is due largely to the activities of microorganisms in the sediment as they mineralize naturally occurring organic materials through biological oxidation. In this process different electron acceptors or sinks such as oxygen, nitrate, sulfate, and carbon dioxide are present. As these sinks are used up, they are reduced to H_2O, NO_2^-

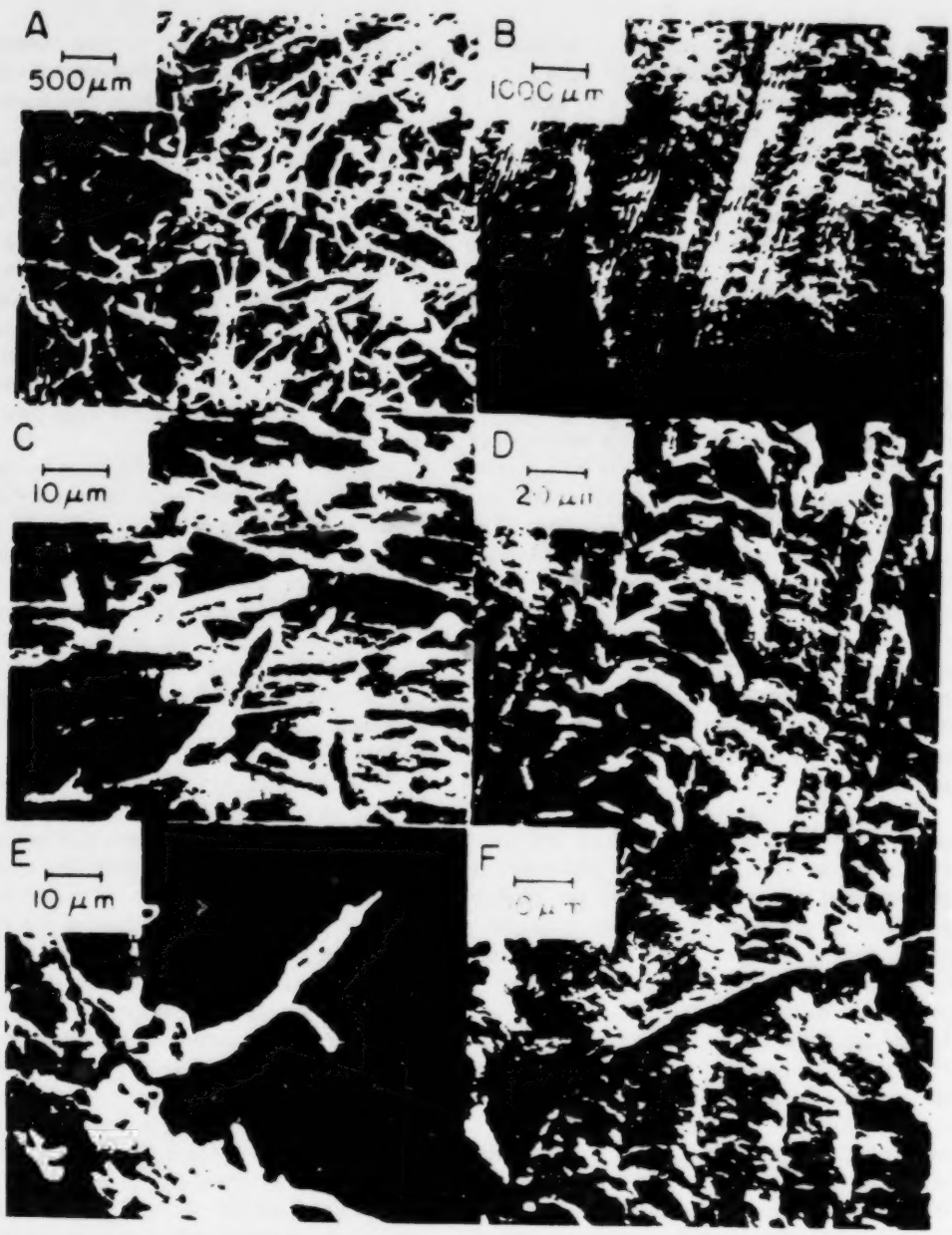

Figure 14 Effect of cadmium on microbial colonization of *Pseudomonas taoda*. (*a*) Control; (*b*) 10μg/l Cd; (*c*) control; (*d*) 10 μg/l Cd; (*e*) control; and (*f*) 5 μg/l Cd. *[Reproduced with permission from Giesy (1978); copyright 1978, Pergamon Press, Ltd.]*

or N_2, SO_2^{2-} or S^{2-}, and methane. As a result of these processes, electrons are transported actively among biological systems through specific chemical reactions of oxidation and reduction and are eventually passed to a final sink (i.e., oxygen, nitrate, sulfate, methane reduction). To measure these activities, researchers commonly add to natural sediments chemicals that are readily oxidized or reduced and whose oxidation-reduction reaction can be readily detected (e.g., by a spectrophotometric monitor). One example is the addition of NADH to sediments. By measuring its oxidation to NAD^+ spectrophotometrically, an indication of the respiratory activity of natural materials may be obtained. The method is known as the dehydrogenase assay since dehydrogenase mediates this process.

Another approach is to add a dye that is readily oxidized and reduced. Various tetrazolium salts, such as triphenyltetrazolium chloride (TTC), react with hydrogen and electrons under anaerobic conditions to form a red formazine compound, which can be measured colorimetrically. The method provides a relative measure of metabolic activity, usually expressed as dehydrogenase activity. Pamatmat and Bhagwat (1973) and Olanczuk and Vosjan (1977) thoroughly characterized the tetrazolium method and found it a quick and simple means of estimating natural rates of anaerobic metabolism by bacteria. It is an integrative method in which activities of all the bacteria growing under anaerobic conditions are considered. Unfortunately, this method has not been used extensively in toxic effects testing. It could be a relatively easy procedure for determining whether a toxic chemical inhibits anaerobic metabolism. Since the method can be used in the field (Pamatmat and Bhagwat, 1973), it is also possible that toxicant-induced stress to an environment can be observed through relative measurements of ETS activity.

Certain pesticides in natural environments may produce toxic residues that not only affect higher organisms but also inhibit the degradation of other pesticides which are not themselves particularly persistent. The use of two different pesticides on cropland may have an adverse effect not obvious with either one alone. Certain persistent xenobiotics (manufactured chemicals) are metabolized in nature only by the nonspecific enzymatic activities found in aquatic bacteria. Many bacteria may not be able to gain energy or carbon from a particular chemical but attack the chemical to some extent because it is similar to a substance readily utilized by the cell. This phenomenon has been referred to as cooxidation or cometabolism (Leadbetter and Foster, 1960). Agriculturally feasible combinations of chemicals may eventually interfere with each other's detoxification through cooxidation or some other more direct process. Engelhardt and Wallnofer (1975) found that a variety of carbamate and organophosphate insecticides inhibited phenylamide herbicide degradation by a pure culture (axenic) of *Bacillus sphaericus*. The effect was compound-specific and occurred at concentrations expected during normal pesticide application to crops. When the activity of crude cell-free preparations of the phenylamide-hydrolyzing acylamidase from cultures of the *Bacillus* species was tested in the presence of the carbamate insecticide, the inhibition was competitive. This means that the carbamate is capable of interfering with the active site of the enzyme or that the enzyme has low specificity. If this occurs in hydrolytic enzyme systems, then a variety of pesticides probably can nonspecifically inhibit degradative processes that require hydrolytic enzymes.

Anderson and Domsch (1980) observed the influence of several pesticides on the degradation of two thiocarbamate herbicides in soil. The degradation of radiolabeled triallate and diallate was followed by examining the parent compound, ^{14}C-labeled bound residues, and mineralization. Bound residues were defined as the radioactivity associated with soil particles that could not be extracted with solvents. Experiments were conducted with flasks containing a bed of soil and a system for trapping carbon dioxide. At the end of

an incubation, the soil was extracted with solvent to recover any parent compound, then combusted to quantitate the radioactivity associated with the soil material (bound residue). After treatment with radioactive triallate and diallate herbicide, the soil samples were treated with several combinations of pesticide, fungicide, and herbicide formulations to simulate typical spray programs for crops (wheat and sugarbeets) in terms of both concentration and chronological application sequence. After 70 d of incubation, minimal influence of the pesticides on diallate degradation was observed (Table 9). Only the pesticide chlorpyrifos significantly inhibited diallate degradation. The effect was cumulative with incubation time. After 70 d, an average of 14% less $^{14}CO_2$ was produced than in the control, and 24% less bound residue was observed. The half-life of diallate (the period after which 50% of the applied concentration could no longer be extracted from the soil in the form of parent compound) was approximately 22 d in the control soils and 30 d in the chlorpyrifos-treated soil. Similar results were obtained with triallate. Chlorpyrifos was applied to the soils as the commercial formulation, and it was possible that one of the formulation components was the toxic agent. To test this, analytical grade chlorpyrifos was applied to soil in the flask and degradation of diallate was again determined. Inhibition was proportional to chlorpyrifos concentration; a concentration of 20 μg/g (ppm) extended the half-life to 70 d. Thus, a formulation component was not the toxic ingredient. Bound residue formation and mineralization were affected to a similar extent. It can be concluded that these pesticides had relatively little effect on the degradation of diallate and triallate. Chlorpyrifos inhibition was not substantial and would probably translate to a minimal effect in the field. However, the experiments illustrate a potential sensitive test of toxicological hazard based on microbial activities.

Pritchard and Bourquin (unpublished information) studied toxic effects of pesticides in estuarine environments. Degradation of methyl parathion was studied in a special biodegradation test system known as an eco-core (Pritchard et al., 1978). This system (Fig. 15) permits the study of degradation while the sediment-water interface (one of the most microbially active areas in aquatic environments) is preserved. An intact sample of water and sediment was collected from a salt marsh with a sterile glass tube. The cores were brought into the laboratory, spiked with ^{14}C-labeled methyl parathion, and stoppered so that air could slowly be bubbled through the water column to maintain proper oxygen concentrations (bubbling does not disturb the sediment-water interface). At different time intervals, replicate cores were analyzed destructively for total parent compound concentration, bound residue formation, and mineralization to CO_2.

Kepone was tested in this system for its effect on the microbial degradation of methyl parathion. Kepone at 2 mg/l was found to significantly inhibit the microbially mediated conversion of methyl parathion to carbon dioxide (Fig. 16). The toxic effect was observed in sediment-water cores from two different estuaries: the Escambia (Range Point salt marsh) and James River estuaries. James River cores were considerably more active in biodegradation of methyl parathion. Although some studies have shown a Kepone concentration in sediments to be as high as 2 ppm, the concentration is variable and patchy. Since normal degradation of methyl parathion, or even other types of organics, occurs in many parts of the James River, it is unlikely that its persistence will be increased significantly. The experimental approach is another method of studying the toxic effects of certain organics to microorganisms and the processes they mediate.

UNIQUE ASPECTS OF THE EFFECTS OF TOXICANTS ON MICROORGANISMS

A variety of toxicology studies differ considerably from the norm, but their creativity and uniqueness merit special attention. These studies are not routine, but they offer new insights that may prove useful in the future development of toxicity

Table 9 Influence of Pesticide Mixtures on Microbial Degradation of [Carbonyl-^{14}C] Diallate in Soil[a,b]

Incubation time (d)	Distribution of ^{14}C-labeled compounds	Chemical treatment[c] and day applied: [^{14}C] Diallate (0)	+ Pyrazon (0)	+ Chlorpyrifos (0)	+ Dimethoate (14)	+ Azinphosmethyl (28)	+ Thiophanate (35)
0	*Soil*[d]						
	Diallate	95.8	–	–	–	–	–
	Bound	3.0	–	–	–	–	–
14	*Atmosphere*						
	Diallate	4.2	3.8	4.6	–	–	–
	CO_2	22.5	22.6	17.9	–	–	–
	Soil						
	Diallate	56.3	58.6	62.1	–	–	–
	Bound	15.6	13.3	13.6	–	–	–
28	*Atmosphere*						
	Diallate	5.8	6.1	7.0	7.1	–	–
	CO_2	36.6	35.7	30.2	28.8	–	–
	Soil						
	Diallate	36.8	36.9	49.8	50.1	–	–
	Bound	19.2	19.3	11.2	12.1	–	–
35	*Atmosphere*						
	Diallate	6.6	6.6	8.1	7.7	8.4	–
	CO_2	37.7	40.4	37.9	30.5	33.9	–
	Soil						
	Diallate	36.3	32.3	41.1	47.6	45.8	–
	Bound	18.4	19.5	11.9	12.6	11.2	–
70	*Atmosphere*						
	Diallate	6.4	7.1	7.1	7.8	8.8	7.8
	CO_2	61.3	62.9	54.6	54.6	49.9	54.3
	Soil						
	Diallate	12.8	10.4	20.8	22.9	26.4	21.7
	Bound	18.5	18.7	15.3	12.9	13.3	15.1

[a] Results, which are averages from duplicate soil cultures and are in percent recovered radioactivity, show the distribution of ^{14}C residues between soil and the atmosphere above the soil. Radioactivity recovered (as percent of applied) was 98.3 ± 1.9 for a total of 38 extractions; lowest recovery was 96.0%.

[b] From Anderson and Domsch (1980).

[c] Diallate and thiophanate applied at 1 μg/g (active ingredient), all other compounds at 2 μg/g.

[d] All soil samples contained traces of water- and benzene-soluble radioactivity. These averaged 1.3–0.4 and 0.2–0.0% of the recovered radioactivity, respectively.

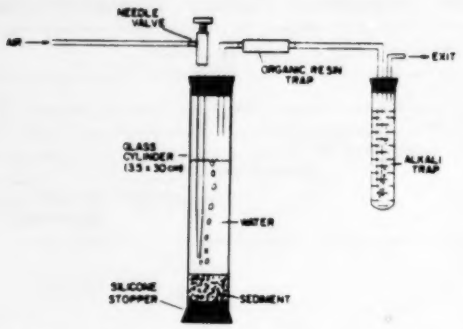

Figure 15 Diagram of eco-core test system. *[From Pritchard et al. (1978).]*

testing. They reflect some fascinating aspects of microbiology and may stimulate new thinking in the ecotoxicology of microbial processes.

Chemotaxis

Bacteria are capable of tactic responses to particular chemicals, including toxicants. Young and Mitchell (1973) showed that toxicants can negatively affect chemotaxis in bacteria. Ordinarily, because bacteria are so small, this type of a response would be difficult to observe. Adler (1969), however, developed a method in which a capillary pipette is filled with a chemical or nutrient source and placed in a dilute culture of bacteria. After a short period of incubation, the mouth of the pipette is covered with bacterial cells (both in and around the pipette). This attraction to a chemical is called positive chemotaxis. When a toxic chemical is included in the nutrient broth in the capillary, the bacteria do not accumulate near the tip or inside; in fact, a distinct clearing zone is often noted around the tip. This is termed negative chemotaxis. Chemicals that neither stimulate nor inhibit chemotaxis are distinct because bacteria around the capillary tip are randomly distributed, and no bacteria are found inside the pipette. Young and Mitchell (1973) showed that bacteria can actively avoid unfavorable areas. Figure 17 shows the distribution of several marine bacterial

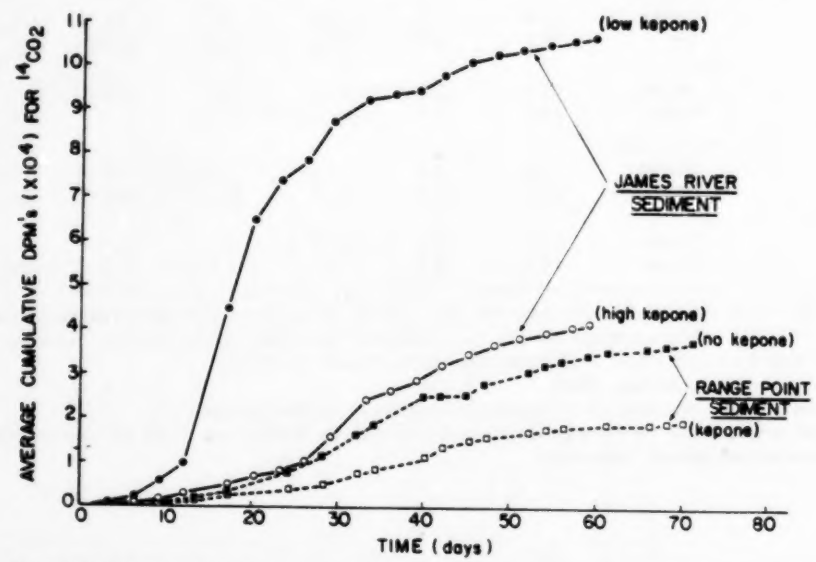

Figure 16 Effects of Kepone on methyl parathion mineralization in eco-cores: (○) 600 ppm and (●) 140 ppm Kepone in James River sediments; (□) 500 ppm Kepone and (■) control sediments from Florida salt marsh. *[From A. W. Bourquin and P. H. Pritchard (unpublished data.)]*

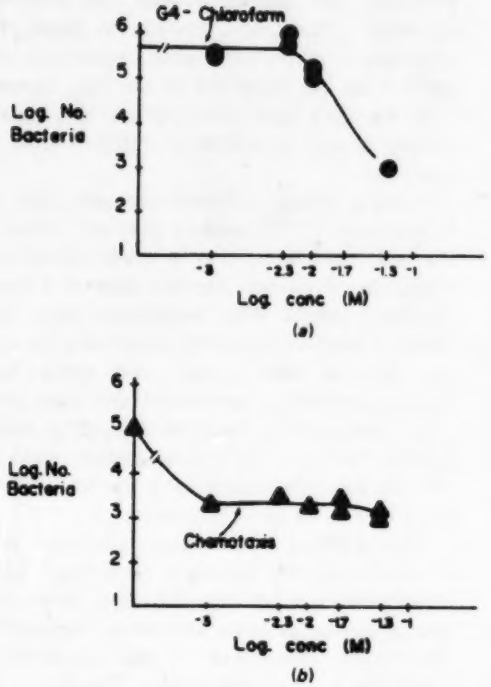

Figure 17 (*a*) Viability of bacterial isolate G4 in the presence of chloroform. (*b*) Number of bacteria entering capillary pipette in the presence of chloroform. *[From Young and Mitchell (1973).]*

isolates in nutrient broth in the presence of chloroform. As the concentration of chloroform is increased, fewer (two orders of magnitude) bacteria are found in the capillary pipette. Comparison of this curve with a survival curve (Fig. 17*a*) shows that chemotaxis occurs at a much lower concentration than that which causes cell death. Mitchell et al. (1972) reported on chemoreception, showing that seawater bacteria are attracted to a wide variety of organic materials, including sugars and amino acids. Isolates obtained from seawater in which the diatom *Skeletonema costatum* was the sole carbon source also showed chemotaxis toward this organism. In addition, they showed that phenol (0.6%), toluene (0.6%), and Kuwait crude oil (concentration unknown) inhibited the chemotactic response to glucose by 80% compared to unexposed control. This tactic response measure has been minimaliy tested with toxicants, yet it has considerable potential as an effects method. It is quick, simple, easy to interpret, and could be used primarily as a screening tool. However, trying to find an ecological meaning for negative taxis is difficult.

Epiphytic Microorganisms

Almost any surface in any body of water has a layer of algae covering it. Invariably associated with the algae and adhering to them is a population of epiphytic bacteria. In this symbiotic relationship, the algae excrete organic materials for bacterial growth, and the bacterial metabolism supplies CO_2 and inorganic nutrients for algal growth. This symbiosis is important for the ecosystem, and it is desirable to know the extent to which a toxicant disturbs this community.

Fry and Ramsay (1977) examined the effect of the herbicide paraquat on the heterotrophic activity of bacteria associated with the leaf surfaces of *Elodea* spp. and *Chara* spp. A washed piece of plant leaf was suspended in a serum bottle containing ^{14}C-labeled glucose and was incubated in the presence of CO_2 trapping agent to measure mineralization (Harrison et al., 1971). Fry and Ramsey (1977) removed the epiphytes from the leaves by vigorously agitating the leaves (scraping the leaves was also successful) in sterile water for 5 min. The leaves were filtered out and the remaining cell suspension used to examine [^{14}C]glucose uptake and mineralization. After thoroughly scraping and cleaning the leaves, it was possible to run controls to determine how much glucose was taken up and mineralized by the plants. The epiphytes accounted for 60% of the glucose taken up by the plant system (Fig. 18). An autoradiographic technique with tritiated glucose showed that only bacteria were actively taking up the glucose, and that attributed to plants (40%) may have been due entirely to bacteria that were not removed.

The effect of paraquat was monitored by

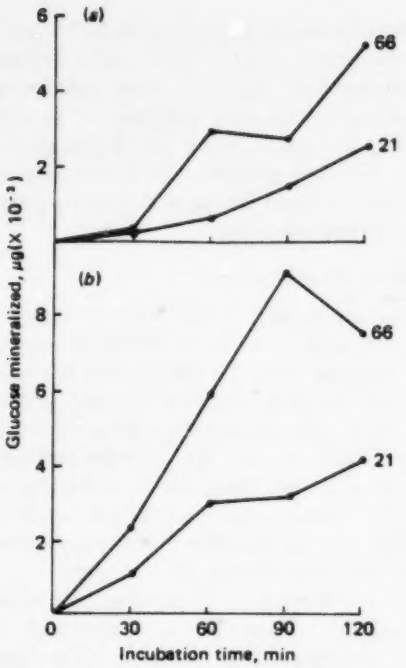

Figure 18 Effects of two concentrations (21 and 66 μg/l) of unlabeled glucose on mineralization of [^{14}C]glucose by epiphytic bacteria: (*a*) *Chara* and (*b*) *Elodea*. *[From Fry and Ramsey (1977).]*

determining the maximum velocity of uptake or mineralization by microorganisms (V_{max}), the glucose concentration that gives half the V_{max}, $K_s + S_n$, (where K_s is the concentration of substrate that gives half the maximal velocity and S_n is the natural glucose concentration), and the time taken for the natural substrate to be mineralized by natural populations (turnover time). Paraquat increased V_{max} and $K_s + S_n$ of bacteria associated with the *Elodea* (Fig. 19). Epiphytic bacteria on the surface of the *Chara* leaves remained unchanged. The herbicide may have stressed the *Elodea*, causing it to release soluble organic compounds which would stimulate growth of the epiphytic bacteria. *Chara* did not release organic materials, presumably because of its resistance to paraquat. The bacterial activity may serve as a secondary indication of toxicity to plants. This technique of estimating kinetic constants is complicated by the variability in the data; however, there was little doubt that epiphytic bacteria were influenced by the release of nutrients from the host plant.

Using a slightly different approach, Bott and Rogenmuser (1978) assessed the toxic effects of the water-soluble fraction of crude oil and used crankcase oil on the attached algae of a typical woodland stream. Algal populations were developed on microscope slides by suspending the slides in a Plexiglas tank through which stream water flowed continually. Herbivores other than protozoans were removed from the slides daily. Once it appeared that the algal population was comparable on each slide, the water-soluble fraction of the oil was added to the tanks continuously.

Several different measurements were employed to determine how the algae responded. Species composition was determined by direct observation with a microscope. Algal biomass was measured by determining chlorophyll *a* and chlorophyll *c* (chlorophyll *c* is more often associated with diatoms). The cell concentration of cyanobacteria was followed through measurements of phycocyanin. Algal material accumulating on the slides was also checked for photosynthetic incorporation of radiolabeled CO_2 into cell material. The water-soluble fraction of these oils reduced algal biomass on the slides (lowered chlorophyll *a*) but caused an increase in cyanobacteria (Table 10). Some studies indicate that blue-green algae are a less desired food source for herbivores; thus their dominance may be an early indicator of adverse effects. Incorporation of radiolabeled CO_2 into cells during photosynthesis was both stimulated and inhibited by the oils. Number 2 fuel oil extracts at high concentrations depressed ^{14}C incorporation. Thus, following the activities of epiphytic algal and bacterial populations associated with surfaces in natural streams appears to offer an excellent approach to toxicity testing.

A variety of other methods can be used to

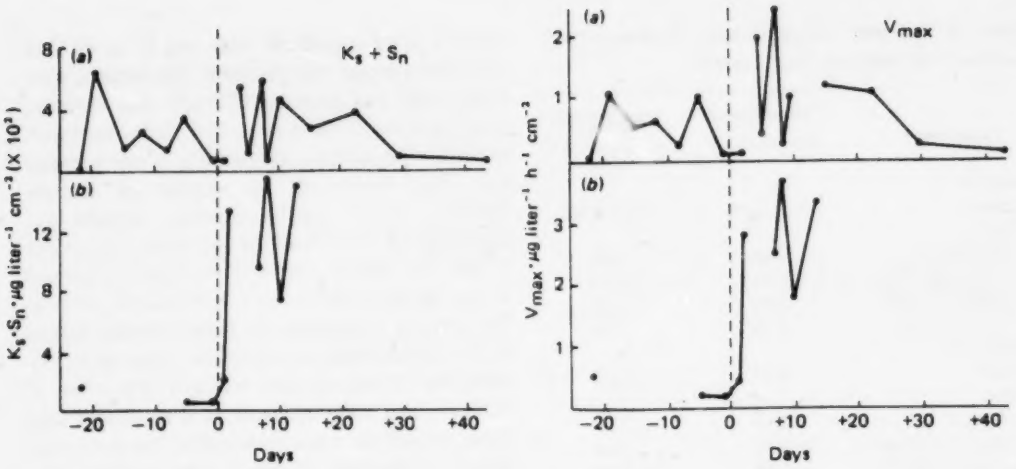

Figure 19 Changes in $K_s + S_n$ and V_{max} during mineralization of glucose by epiphytic bacteria of *Chara* and *Elodea* before and after treatment with paraquat. Only results having positive regressions are plotted. (*a*) *Chara* and (*b*) *Elodea*. Paraquat was applied on d 0. *[From Fry and Ramsey (1977).]*

monitor the effects of toxic chemicals on epiphytic microbial populations. Andrews and Kenerley (1978) plated out on agar medium the bacteria and fungi associated with apple leaf surfaces. Agar plates were inoculated with leaf washings, with spores that fell from the leaf surface, or by imprinting leaves on the agar. Light and electron microscopy were used to examine morphological and community structure changes on the leaf surfaces. Lactic acid-type bacteria and fluorescent pseudomonads were most depressed by a series of pesticides applied as in crop spraying. Although apple leaves were used, the same techniques could be applied to aquatic plants and vegetation.

Most toxicological studies on aquatic bacteria are performed with bacteria found in the water column, undoubtedly the easiest and most accessible populations to study. However, epiphytic microorganisms are an important part of the microbial community and should be considered for study whenever possible. In salt-marsh environments and wetland areas, epiphytic bacteria may be more important in the mineralization of organic carbon from plants to CO_2 and inorganic nutrients than bacteria of the water column. Giesy (1978) showed that the epiphytes on rock surfaces are the major mineralizers in a stream ecosystem.

Incidence of Plasmid-containing Bacteria

The very low levels of organic and inorganic nutrients in marine environments may limit the growth and survival of many microorganisms. Consequently, marine bacterial populations maintain a delicate balance between genetic flexibility and metabolic capabilities. Maintenance of this balance may result in preferential loss of certain nonessential genetic elements, particularly extrachromosomal DNA or plasmids (Smith & Gardner, 1970). By studying the incidence of plasmids in marine bacterial populations, it may be possible to show that a pollutant is changing the nutrient conditions of the environment and consequently changing the genetic makeup of the indigenous bacteria.

Hada and Sizemore (1981) discussed effects of crude oil pollution on microorganisms which could

Table 10 Pigment Ratios of Algal Communities: Summer and Autumn Experiments[a]

Experiment	Chlorophyll *c* / chlorophyll *a*	Phycocyanin / chlorophyll *a*
Summer		
Start	0.11–0.15	0.03–0.05
End		
Control	0.08	0.06
Used crankcase	0.14	0.12
Nigerian crude	0.07	0.12
No. 2 fuel oil	0.15	0.33
Autumn		
Start	0.12	0.08
End		
Control	0.14	1.33
Used crankcase	0.17	0.96
Nigerian crude	0.13	0.93
No. 2 fuel oil	0.07	2.13

^{14}C incorporation (dpm/μg of chlorophyll *a*) by algal communities exposed to water-soluble fractions of oils[b]

Extract exposure	July 19	August 13	August 24
Control	1869	1246	1890
Crankcase	1418	1542	1262
Nigerian crude	1369	1112	972
No. 2 fuel oil	1384	3691	927

[a] From Bott and Rogenmuser (1978).
[b] Corrected for dark fixation and excretion. Single determinations $\bar{x} \pm SD$ for 7/19 (P^s) = 1510 ± 240 (16%).

be applicable in future assays for other toxic pollutants. They determined the incidence of DNA plasmids (extrachromosomal pieces of DNA found in the cytoplasm of bacterial cells) in marine *Vibrio* spp. in relation to the pollution history of the sampling area. The marine *Vibrio* spp. are gram-negative, facultatively anaerobic, rod-shaped bacteria common in many estuarine and marine environments. They are associated with diseases of humans and appear to be the "enteric bacteria" of the sea (Bauman and Bauman, 1977). Thus their survival could be coupled to pollution stress in the marine environment.

Hada and Sizemore (1981) isolated marine *Vibrio* spp. throughout the year from sampling stations in an unpolluted area and in an oil-field area near a production platform. The bacteria were lysed with Brij solution (Clewell and Helinski, 1969) and the DNA in the lysate precipitated with ethanol. The number of plasmids in this preparation was determined by agarose gel electrophoresis, where the components separate out according to their mobility and molecular weight (Table 11). The incidence of plasmids was greater at the oil-field stations than the control stations. The number of plasmids per isolate was also higher at the oil-field stations (compared to the controls). Since the oil-field stations receive a large influx of nutrients and toxic materials, it is speculated that these conditions were responsible for the multiplicity of plasmids. If this is correct, plasmids in microorganisms may serve as indicators of water

Table 11 Incidence of *Vibrio* spp. and Plasmid Containing *Vibrio* spp. for Gas and Oil-Field Stations and Control Site during Four Quarterly Cruises[a]

	Oil-field stations		Control stations	
Season	No. of *Vibrio* spp.	Percent *Vibrio* spp. with plasmids	No. of *Vibrio* spp.	Percent *Vibrio* spp. with plasmids
Fall	58	45	90	21
Winter	42	30	3	66
Spring	35	23	6	33
Summer	168	35	38	21

Plasmid multiplicity of plasmid-containing strains

	Percent of plasmid-containing strains	
Plasmid bands per strain	Oil-field isolates	Control station isolates
1	39.6	71
2	24.5	16
3	16.0	0
4	3.8	13
5	2.8	0
6	7.5	0
7	5.7	0

[a] From Hada and Sizemore (1981).

pollution. The results also suggest that the genetic and metabolic capabilities of these organisms may have changed, making it possible for some natural processes to be short-circuited or supplemented.

Bioaccumulation in Bacteria

Ko and Lockwood (1968) showed that chlorinated hydrocarbon pesticides (dieldrin, DDT, and the fungicide pentachloronitrobenzene) were readily accumulated by pure cultures of soil fungi and actinomycetes. Grimes and Morrison (1975) extended the observation to aquatic bacterial isolates. Pure cultures were grown in nutrient broth, washed by centrifugation, and resuspended in various pesticides (in acetone carrier) at a concentration of 0.1 μg/l. At specific time intervals, subsamples were removed and centrifuged; the concentration of the pesticide in the water was determined by gas chromatography. These pesticides were bioconcentrated (i.e., the ratio of pesticide in the cell to pesticide in the water increased) by factors ranging from 10 for lindane to as much as 59,000 for chlordane. Pesticides were ranked by bioconcentration potential: the more water-insoluble the pesticide, the higher the bioconcentration factor. Once inside the cells (equilibrium was reached within 15 min), the pesticides were difficult to extract. Extraction from the cells was also inversely related to water solubility. Because of the affinity of the insoluble pesticides for lipid-containing materials in the cell, pesticides would be expected to localize in cellular membranes. However, Grimes and Morrison (1975) found that the stalked bacterium *Caulobacter vibrioides*, which has the greatest cellular lipid content among bacteria, had the highest bioconcentration values.

Geller (1979) showed that bioaccumulation (at least for the herbicide atrazine) is a function of cell surface area. He observed that physiological and morphological groups of bacteria with different cellular volumes took up different amounts of atrazine. In contrast to the results of Grimes and Morrison (1975), Geller found that atrazine was readily taken up by the cells, but noted that cell aggregates observed after extended incubation periods in liquid culture were less effective in absorbing atrazine. This might have resulted from a decreased surface area, or it might mean that actively growing cells (those not in aggregates) are the best bioconcentrators. In studies with the common aquatic myxobacterium *Cytophaga*, the high bioconcentration of atrazine was related to the extensive slime layer typically deposited on the surface of these cells. These studies indicate a general nonspecific mechanism of bioaccumulation which is more dependent on the biochemical composition of the cells than on a physiological or active transport mechanism. Since bacteria are the initial step in the food chain of aquatic systems, bioaccumulation may be a principal path by which toxic chemicals enter food chains, leading to damage and stress to ecosystems. Whether this is the case depends on how well the role of bacteria in the food chain can be described. It may also be possible that bacteria that accumulate pesticides can be used as biological sponges. Under controlled laboratory conditions, bacteria flocs could be used to remove pesticides from aquatic environments (Speidel et al., 1972).

ADVERSE EFFECTS OF MICROORGANISMS ON TOXIC SUBSTANCES

Toxicity of a chemical in an aquatic environment is tightly coupled to its chemical and biological fate. Some very toxic chemicals are innocuous in the environment because their degradation to nontoxic by-products is rapid. Biodegradation is one of the principal fates of these chemicals, and humans depend on the metabolic diversity of microbial populations to ensure that many synthetic organic materials do not accumulate in the environment. The biodegradation capabilities of bacteria have been overchallenged. Chemicals have been synthesized with structures that the enzymatic machinery of microorganisms cannot attack at significant rates, leading to major pollution problems with chemicals such as DDT, Kepone, and polychlorinated biphenyls (PCBs).

Biodegradation mechanisms and capabilities of aquatic organisms are discussed in Chapter 18. It is emphasized here, however, that biodegradation may generate a product more toxic than the parent compound. Although this is not well documented in nature, there are enough examples to concern microbiologists and to warrant studies of the toxicity of degradation products. Mercury is probably the most publicized example of a substance that was more toxic after microbial transformation. The anaerobic activities of microorganisms in sediments caused methylation of mercury (Bisogni and Lawrence, 1975; Olsen and Cooper, 1976). As a result, the mercury was made more water-soluble and was mobilized from the sediment and more thoroughly mixed with the water column. More toxic forms of the element (methyl and dimethyl mercury) resulted. Sayler et al. (1975) showed that bacteria, through bioaccumulation of mercury and consumption by higher organisms, can enhance the concentration of mercury in aquatic food chains. Methylation of other elements (cadmium, arsenic, tin) by microorganisms can also potentially produce a more toxic by-product.

The fate of the antimicrobial agent pentachlorobenzyl alcohol caused major concern in Japan. This chemical was applied to rice plants to control a common fungus. However, once the plants died and decayed in the soil, pentachlorobenzyl alcohol was converted to pentachlorobenzoic acid. This metabolic product was toxic to plants but no longer toxic to fungi. Pesticides such as DDT, mirex, heptachlor, and dieldrin can be slowly attacked by microorganisms and other degradative processes. The degradation products are usually resistant to further degradation and transformation, and their persistence and toxicity in the environment are of concern.

Crude oil contains many polynuclear aromatic hydrocarbons that are slowly degraded and could yield intermediate degradation products that are carcinogenic and mutagenic. Organophosphate insecticides have P=S bonds and could be oxidized by bacteria to corresponding P=O (oxone) derivatives, which have considerably greater mammalian toxicity. Bacteria participate in *N*-alkylation and nitrosamination leading to the formation of nitrosamines from a variety of common precursors such as secondary amines and nitrites. Nitrosamines are powerful carcinogens and mutagens. Although many of these potential degradation products are not toxic to bacteria, bacterial activity leads to their formation.

SUMMARY

Microorganisms are an integral part of the nutrient cycling and energy flow processes of aquatic ecosystems. Their degradative and mineralization capabilities maintain the carbon balance of the ecosystem and also permit humans to dispose of synthetic wastes in aquatic environments. These environments have a certain assimilatory capacity for the wastes, and their biodegradation potential determines the magnitude of this capacity. Humans must not exceed this capacity. The generation of persistent or nonbiodegradable chemicals show how easily this capacity can be abused. The input of chlorinated hydrocarbons into the environment exceeded their biological detoxification, and major ecological damage resulted. Had the assimilatory capacity been properly assessed, environmental abuse might have been avoided.

The toxicity of chemicals to bacteria must be carefully observed and researched. If a new synthetic chemical is developed and allowed to pollute the environment, some microbial processes could be disturbed, and this could eventually have an indirect but catastrophic effect on the entire ecosystem. Severe inhibition of nitrification can affect plant communities. If photosynthetic production drops, the effect ripples throughout the ecosystem.

Bacteria and fungi and the processes they catalyze are fairly resistant to inhibition by synthetic chemicals. A small perturbation in one part of the environment can often be overcome because of the ubiquity of microorganisms and their great metabolic diversity. Nonetheless, toxi-

cologists should incorporate microorganisms into testing schemes, since they may be potential indicators of environmental damage.

The importance of using a wide diversity of toxicity tests for microorganisms cannot be overemphasized. Rapid methods for assessing chemical toxicity to microorganisms are needed for detecting chemicals that are potentially toxic to the ecosystem. Continued research in basic microbial ecology should help fill this need.

LITERATURE CITED

Adler J: Chemoreception in bacteria. Science 166:1588–1597, 1969.

Anderson JP, Domsch KH: Influence of selected pesticides on the microbial degradation of ^{14}C-triallate and ^{14}C-diallate in soil. Arch Environ Contam Toxicol 9:115–123, 1980.

Andrews JH, Kenerley CM: The effects of a pesticide program on non-target epiphytic microbial populations of apple leaves. Can J Microbiol 24:1058–1072, 1978.

Bauman P, Bauman L: Biology of the marine enterobacteria: *Beneckea* and *Photobacterium*. Annu Rev Microbiol 31:39–61, 1977.

Billen G: Evaluation of nitrifying activity in sediments by dark ^{14}C-bicarbonate incorporation. Water Res 9:51–57, 1975.

Bisogni JJ, Lawrence AW: Kinetics of mercury methylation in aerobic and anaerobic aquatic sediments. J Water Pollut Control Fed 47:135–152, 1975.

Bohool BB, Wiebe WJ: Nitrogen fixing communities in an intertidal ecosystem. Can J Microbiol 24:923–938, 1978.

Bott TL, Rogenmuser K: Effects of No. 2 fuel oil, Nigerian crude oil, and used crankcase oil on attached algal communities: Acute and chronic toxicity of water-soluble constituents. Appl Environ Microbiol 36:673–682, 1978.

Bourquin AW, Pritchard PH, Mahaffey WR: Effects of Kepone on estuarine microorganisms. Dev Ind Microbiol 19:489–497, 1978.

Breazeal FW, Camper ND: Effect of selected herbicides on bacterial growth rates. Appl Environ Microbiol 23:431–432, 1972.

Charyulu PBB, Ramakrishna C, Rao VR: Effect of 2-aminobenzimidazole on nitrogen fixers from flooded soil and their nitrogenase activity. Bull Environ Contam Toxicol 25:482–486, 1980.

Clewell DB, Helinski DR: Supercoiled circular DNA-protein complex in *Escherichia coli*: Purification and induced conversion to an open circular DNA form. Proc Natl Acad Sci USA 62:1159–1166, 1969.

Colwell RR, Morita RY: Effect of the ocean environment on microbial activities. In: Proceedings of the 2d U.S.-Japan Conference on Marine Microbiology. Baltimore: University Park Press, 1974.

DaSilva EJ, Henriksson LE, Henriksson E: Effect of pesticides on blue-green algae and nitrogen fixation. Arch Environ Contam Toxicol 3:193–204, 1975.

Daley RJ, Hobbie JE: Direct counts of aquatic bacteria by a modified epifluorescence technique. Limnol Oceanogr 20:875–882, 1975.

Englehardt G, Wallnofer, PR: Inhibition of phenylamide hydrolysis by *Bacillus sphaericus* with methyl carbamate and organophosphorous insecticides. Appl Environ Microbiol 29:717–721, 1975.

Fry JC, Brooker MP, Thomas PL: Changes in the microbial populations of a reservoir treated with the herbicide paraquat. Water Res 7:395–407, 1973.

Fry JC, Ramsey AJ: Changes in the activity of epiphytic bacteria of *Elodea canadensis* and *Chara vulgaris* following treatment with the herbicide paraquat. Limnol Oceanogr 22:556–561, 1977.

Geller A: Sorption and desorption of atrazine by three bacterial species isolated from aquatic systems. Arch Environ Contam Toxicol 8:713–720, 1979.

Giesy JP: Cadmium inhibition of leaf decomposition in an aquatic microcosm. Chemosphere 6:467–475, 1978.

Goulder R, Blanchard AS, Sanderson PL, Wright B: Relationships between heterotrophic bacteria and pollution in an industrial estuary. Water Res 14:591–601, 1980.

Grimes DJ, Morrison SM: Bacterial bioconcentration of chlorinated hydrocarbon insecticides from aqueous systems. Microb Ecol 2:43–59, 1975.

Guthrie RK, Cherry DS, Ferebee RN: The effects of diuron on bacterial populations in aquatic environments. Water Resour Bull 10:304–310, 1974.

Hada HS, Sizemore RK: Incidence of plasmids in marine *Vibrio* spp. isolated from an oil field in the northwestern Gulf of Mexico. Appl Environ Microbiol 41:199–202, 1981.

Harrison MJ, Wright RT, Morita RY: Method for measuring mineralization in lake sediments. Appl Microbiol 21:698–702, 1971.

Herbert RA: Heterotrophic nitrogen fixation in shallow estuarine sediments. J Exp Mar Biol Ecol 18:215–225, 1975.

Hodson RE, Azam F, Lee RF: Effects of four oils on marine bacterial populations: Controlled ecosystem pollution experiment. Bull Mar Sci 27:119–126, 1977.

Holm-Hansen O, Booth CR: The measurement of adenosine triphosphate in the ocean and its ecological significance. Limnol Oceanogr 11: 510–519, 1966.

Jones RD, Hood NA: The effects of organophosphorous pesticides on estuarine ammonia oxidizers. Can J Microbiol 26:1296–1299, 1980.

Ko WH, Lockwood JL: Accumulation and concentration of chlorinated hydrocarbon pesticides by microorganisms in soil. Can J Microbiol 14:1075, 1968.

Kogure K, Simidu U, Taga N: A tentative direct microscopic method for counting living marine bacteria. Can J Microbiol 25:415–420, 1979.

Koike J, Hattori A: Simultaneous determinations of nitrification and nitrate reduction in coastal sediment by an ^{14}N dilution technique. Appl Environ Microbiol 35:853–857, 1978.

Leadbetter ER, Foster JW: Bacterial oxidation of gaseous alkanes. Arch Microbiol 35:92–194, 1960.

Lee KK, Watanabe J: Problems of the acetylene reduction technique applied to water-saturated paddy soils. Appl Environ Microbiol 34:654–660, 1977.

Mahaffey WR, Pritchard PH, Bourquin AW: Effect of Kepone on growth and respiration of several estuarine microorganisms. Appl Environ Microbiol 43:1419–1424, 1982.

Matthews RA, Kondratieff PF, Buikema AL: A field verification of the use of the autotrophic index in monitoring stress effects. Bull Environ Contam Toxicol 25:226–233, 1980.

Menzel DW, Case J: Concept and design: Controlled ecosystem pollution experiment. Bull Mar Sci 27:1–7, 1977.

Mitchell R, Fogel S, Chet I: Bacterial chemoreption: An important ecological phenomenon inhibited by hydrocarbons. Water Res 6:1137–1140, 1972.

Olanczuk-Neyman KM, Vosjan JH: Measuring respiratory electron-transport-system activity in marine sediments. Neth J Sea Res 11:1–13, 1977.

Olsen BH, Cooper RC: Comparison of aerobic and anaerobic methylation of mercuric chloride by San Francisco Bay sediments. Water Res 10:113–116, 1976.

Orndorff SA, Colwell RR: Effect of Kepone on estuarine microbial activity. Microb Ecol 6:357–368, 1980.

Pamatmat MM, Bhagwat AM: Anaerobic metabolism in Lake Washington sediments. Limnol Oceanogr 18:611–627, 1973.

Pritchard PH, Bourquin AW, Frederickson J'L, Maziarz T: System design factors affecting environmental fate studies in microcosms. In: Proceedings of the Workshop: Microbial Degradation of Pollutants in Marine Environments, edited by AW Bourquin, PH Pritchard, pp. 251–272. EPA-600/9-79-012, U.S. Environmental Protection Agency, 1978.

Sayler GS, Nelson JD, Colwell RR: Role of bacteria in bioaccumulation of mercury in the oyster, *Crassostrea virginica*. Appl Microbiol 30:91–96, 1975.

Smith DH, Gardner P: The ecology of R factors. N Engl J Med 202:161–162, 1970.

Speidel HK, Bourquin AW, Mann JE, Fair JF, Bennett EO: Microbial removal of pesticides from aqueous environments. Dev Ind Microbiol 13:277–282, 1972.

Trevors JT, Basaraba J: Toxicity of benzoquinone and hydroquinone in short term bacterial bioassays. Bull Environ Contam Toxicol 25:672–675, 1980.

Trudgill PW, Widdus R, Ross JS: Effects of organochlorine insecticides on bacterial growth, respiration and viability. J Gen Microbiol 69:1–5, 1971.

Ware GW, Roan CC: Interaction of pesticides with aquatic microorganisms and plankton. Residue Rev 33:15–45, 1970.

Widdus R, Trudgill PW, Turnell DC: Effects of technical chlordane on growth and energy metabolism of *Streptococcus faecalis* and *Mycobacterium phlei*: A comparison with *Bacillus subtilis*. J Gen Microbiol 69:21–23, 1971.

Wright RJ: Measurement and significance of specific activity in the heterotrophic bacteria of natural waters. Appl Environ Microbiol 36:297–305, 1978.

Young CY, Mitchell R: Negative chemotaxis of marine bacteria to toxic chemicals. Appl Microbiol 25:972–975, 1973.

Zar JH: Biostatistical Analysis, pp. 151–162. Englewood Cliffs, N.J.: Prentice-Hall, 1974.

SUPPLEMENTAL READING

Anderson JR: Some method for assessing pesticide effects on non-target soil microorganisms and their activities. In: Pesticide Microbiology, edited by IR Hill, SJL Wright, pp. 247–533. New York: Academic, 1978.

Bourquin AW, Pritchard PH: Workshop: Microbial Degradation of Pollutants in Marine Environments, EPA-600 19/79-012; p. 545. U.S. Environmental Protection Agency, 1979.

Colwell RR, Morita RY: Effect of the ocean environment on microbial activities. In: Proceedings of the 2d U.S.-Japan Conference on Marine Microbiology. Baltimore: University Park, 1972.

Lynch JM, Poole NJ: Microbial Ecology: A Conceptual Approach. New York: Wiley, 1979.

Mitchell R: Water Pollution Microbiology, vol. 1. New York: Wiley-Interscience, 1972.

Pfister RM: Interactions of halogenated pesticides and microorganisms–a review. In: Microbial Ecology, edited by AI Laskin, H Lechevalier, pp. 1–33. Cleveland: CRC Press, 1972.

Rheinheimer G: Aquatic Microbiology. London: Wiley, 1971.

Tu, CM, Miles JRW: Interactions between insecticides and soil microbes. Residue Rev 64:17–65, 1976.

Ware GW, Roan CC: Interaction of pesticides with aquatic microorganisms and plankton. Residue Rev 33:15–45, 1970.